MATERNAL PROTEIN RESERVES, DIET AND LACTATIONAL PERFORMANCE IN RATS

by

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DECLARATION

I declare that this thesis has been composed by myself. The experimental work and analyses were carried out by myself, with the assistance of other people as indicated in the acknowledgements. The work in this thesis has not been submitted for any other degree or qualification.

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ABSTRACT

The importance of tissue protein reserves to lactating females attempting to sustain milk output under conditions of severe dietary protein restriction was investigated using rats. Four experiments were carried out to study the effect of variation in repletion of tissue protein reserves on lactational performance, rates of body protein mobilisation and changes in tissue protein metabolism involved in promoting protein mobilisation. The extent to which body protein reserves were capable of maintaining milk quantity and quality under such conditions was also considered.

The lactational performance of multiparous, female Sprague-Dawley rats, offered isoenergetic diets (21 MJ GE/kg DM), was assessed from growth of a standardised litter of 12 pups. Variation in repletion of protein reserves at parturition was achieved by applying a period of protein restriction during the latter half of gestation. Changes in body composition were estimated from carcass analysis and rates of protein mobilisation were derived from serial slaughter experiments. Tissue protein synthesis was estimated *in vivo* using a flooding dose of [^{3}H] phenylalanine and tissue Na^+,K^+-ATPase activity was measured polarographically *in vitro* . Milk samples were obtained following injection of oxytocin.

Females offered a high protein diet (215 g CP/kg DM) during lactation exhibited an increase in both feed intake and lactational performance while not utilising their body protein stores. However, in rats offered imbalanced feeds (low protein/high energy) such an increase in intake was not apparent and dams were forced to draw upon their endogenous protein reserves in an attempt to sustain milk production. Between 15 and 22 % of body protein was lost by dams assumed to be "Fully" protein replete at parturition. When dietary protein was limiting, reductions in the size of the protein reserve had a significant impact on a female's ability to sustain milk production, and dams which were initially "Fully" replete supported greater ($P<0.05$) litter growth during early lactation, due to a greater endogenous protein supply and feed intake ($P<0.05$), than their "Depleted" contemporaries. Prior depletion of

body protein reserves had no significant impact on lactational performance when an adequate supply of dietary protein was provided.

The use of body protein reserves during lactation could not maintain either milk quantity or quality at the level of better fed females and throughout lactation milk protein and fat contents were considerably altered ($P<0.05$) compared to that of well nourished dams. Such changes limit the applicability of milk yield prediction equations for undernourished dams.

Rates of protein mobilisation during lactation varied with the degree of protein inadequacy and ranged from 0 g/d in dams on a 215 gCP/kg DM feed to 0.49 g/d (0.01 d^{-1}) and 1.01 g/d (0.021 d^{-1}) in dams offered a 150 and 90 gCP/kg DM diet respectively.

A period of protein undernutrition during early lactation did not prevent dams from improving their lactational performance ($P<0.01$) in response to an increase in the dietary protein supply and this was favoured by the maintenance of the mammary cell population during this period. The data suggest that the mammary cell population is quite sensitive to nutritional status in rats.

Tissue protein mobilisation during lactation was associated with a dramatic increase in muscle protein degradation, to 13.0 %/d, while the decline in protein synthesis was of less importance, although this was reflected in a reduced Na^+,K^+-ATPase activity ($P<0.05$). Mammary protein synthesis (FSR and ASR) increased during lactation ($P<0.05$) in dams offered a high protein/high energy feed, although this was prevented by dietary protein restriction. Whilst liver FSR was less sensitive to dietary protein content, changes in liver ASR reflected the effect of dietary treatment on liver size ($P<0.001$).

In vivo rates of mammary protein synthesis were adversly affected by the use of exogenous oxytocin in the milking procedure used immediately before estimation, although rates of muscle and liver protein synthesis were unaffected.

When low dietary protein concentration constrained feed intake there was also a considerable mobilisation of body fat and it appeared that under these conditions intake

constraint was associated with the impact of a nutrient imbalance on a dams ability to dispose of surplus ("imbalanced") nutrients.

Nutrient balances developed for severely protein restricted dams during lactation indicated that tissue protein reserves support lactation not only through the provision of endogenous amino acids but also by allowing an increased feed intake.

CONTENTS

ABBREVIATIONS

ADP	- Adenosine Diphosphate
ASR	- Absolute Synthesis Rate
ATP	- Adenosine Triphosphate
ATPase	- Adenosine Triphosphatase
BHT	- Butylated Hydroxy Toluene
CHO	- Carbohydrate
CP	- Crude Protein
DM	- Dry Matter
DNA	- Deoxyribonucleic acid
DPM	- Disintegrations per minute
ECF	- Extra Cellular Fluid
FDR	- Fractional Degradation Rate
FFM	- Fat Free Milk
FSR	- Fractional Synthesis Rate
GE	- Gross Energy
HC	- Heat Capacity
HEPES	- N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
$[^3H]$	- Tritium
KRB	- Krebs-Ringer Bicarbonate buffer
MEM	- Minimal Essential Medium
Ks	- Fractional Synthesis Rate
MW	- Molecular Weight
NAD^+	- Nicotinamide Adenine Dinucleotide (oxidized)
NADH	- Nicotinamide Adenine Dinucleotide (reduced)
NEFA	- Non Esterified Fatty Acid
NRC	- National Research Council
OM	- Organic Matter
PD	- Potential Difference
RNA	- Ribonucleic acid
S_A	- Free Phenylalanine Specific Activity
S_B	- Protein Bound Phenylalanine Specific Activity
SD	- Standard Deviation
SEM	- Standard Error of the Mean
t	- Incorporation time
TCA	- Trichloroacetic acid

CHAPTER ONE

INTRODUCTION and LITERATURE REVIEW

Lactation is the sole characteristic by which mammals are distinguished from other vertebrates and relies on the metabolic activity of a specialised organ, the mammary gland. Following parturition, lactation supports the growth and development of the new-born through the provision of milk that contains a balanced supply of nutrients including carbohydrate (lactose), protein, fat, vitamins and minerals. The available information on mammalian milk composition suggests that although qualitatively the milk of different species contains the same basic constituents, quantitatively there is considerable species variation (Table 1.1). The major milk components are synthesised within the gland from precursors (glucose, amino acids, triglycerides and free fatty acids) derived from the blood supply.

Table 1.1. The average concentration of the major constituents in the milk of a number of mammalian species.

SPECIES	FAT (g/l)	CASEIN (g/l)	MILK SERUM Protein (g/l)	LACTOSE (mM)	CALCIUM (mM)
Cow	37	28	6	133	30
Goat	45	25	4	114	22
Sheep	74	46	9	133	58
Pig	68	28	20	153	104
Horse	19	13	12	172	17
Man	38	4	6	192	7
Guinea-Pig	39	66	15	83	41
Rabbit	183	104	32	60	214
Rat	103	64	20	90	80

Davies *et al.* (1983)

The lactating mammary gland is a highly active organ and its nutrient output in milk may actually exceed the nutrient utilisation of the rest of the body. In modern dairy systems, high yielding cows often have peak milk yields of 30 - 40 kg/d which means the daily secretion of at least 2 kg of lactose and 1.5 kg each of protein and fat (Mepham 1987). Milk production is even more impressive in smaller mammals and in rodents the daily output can be as much as 20 % of total body weight (Grigor *et al.* 1987a). Lactation therefore imposes an enormous demand on maternal metabolism and mammals overcome this considerable metabolic challenge through a number of adaptations.

Lactating females attempt to satisfy these increased requirements primarily by elevating their feed intake and this has been confirmed for cattle (Garnsworthy 1988), pigs (Mullan *et al.* 1989a) and rodents (Naismith *et al.* 1982,Williamson 1980). This increased nutrient supply is supported by an increase in the size and absorptive capacity of the digestive tract, while also promoting considerable hypertrophy of the liver and mammary gland in both ruminants (Vernon 1988) and rodents (Williamson 1980).

Since the precursors of milk constituents can also be used as nutrients by other body tissues, it is possible that, despite the increased nutrient supply, this competition for available nutrients could be deleterious to milk secretion. However, it can be concluded that lactation is accorded a high priority, and it is thought to proceed at the expense of other biological functions even to the creation of disease situations (Bauman *et al.* 1983) e.g. ketosis. Therefore in-addition to the increased feed intake, lactation is associated with co-ordinated changes in maternal metabolism such that a high proportion of available nutrients (triglycerides, glucose, amino acids) are partitioned away from peripheral tissues (adipose tissue, muscles) and towards the mammary gland (Vernon 1989). The recognised changes in tissue metabolism during lactation in the rat are shown in Table 1.2. Such partitioning of available nutrients to selective organs during lactation is further amplified in ruminants (Davis *et al.* 1985, Lomax *et al.* 1983) and rodents (Chatwin *et al.* 1969) by an increase in cardiac output and blood flow to the digestive tract, liver and mammary gland. However, despite this co-ordinated shift in maternal metabolism, there are times during lactation when the nutrients supplied from feed intake cannot support the increased demands of the mammary gland. Such a short-fall between supply and demand is often observed in the dairy cow during early lactation when appetite increases more slowly than milk yield (Bauman *et al.* 1980) and when severely imbalanced diets (protein:energy) are offered to lactating rodents (Friggens 1990, Naismith *et al.* 1982). Under such conditions, the nutrient pool, from which milk precursors are derived, can also be supplemented by the mobilisation of maternal reserves of fat, protein and minerals.

Table 1.2. Metabolic adaptations to lactation in tissues of the lactating rat.

TISSUE	PROCESS	CHANGE
Mammary Gland	Glucose utilisation	Increased
	Lactose synthesis	Increased
	Protein synthesis	Increased
	Amino acid utilisation	Increased
	Lipogenesis	Increased
	Fatty acid esterification	Increased
	Lipid uptake	Increased
Liver	Glucose utilisation	Increased
	Protein synthesis	Increased
	Lipogenesis	Increased
	Fatty acid esterification	Increased
	Ketogenesis	Decreased
White Adipose Tissue	Glucose utilisation	Decreased
	Lipogenesis	Decreased
	Lipid uptake	Decreased
	Fatty acid esterification	Decreased
	Lipolysis	Increased
Brown Adipose Tissue	Glucose utilisation	Decreased
	Lipogenesis	Decreased
	Thermogenesis	Decreased
Skeletal Muscle	Glucose utilisation	Decreased
	Protein Synthesis	?
	Protein Degradation	?

? Changes unknown
Source : Williamson (1980), Williamson *et al.* (1986), Sampson *et al.* (1986), Trayhurn *et al.* (1982), Vernon (1989)

From the above discussion it is apparent that successful lactation not only requires alterations in mammary gland activity but perhaps more importantly, co-ordinated changes in the metabolism of maternal tissues such that the mammary gland is provided with the bulk of the available nutrients. Alterations in maternal metabolism in support of the new physiological state have therefore been proposed to be under homeorhetic control (Bauman *et al.* 1980).

The adaptations in tissue metabolism that ensures the preferential utilisation of available nutrients by the mammary gland are ultimately co-ordinated by alterations in the female's endocrine status. The major changes in serum hormone concentrations during lactation in rodents and ruminants are described in Table 1.3.

Table 1.3. Changes in serum hormone concentrations in ruminants and rodents during lactation

HORMONE	RODENTS	RUMINANTS
Insulin	Decreased	Decreased
Prolactin	Increased	Increased
Growth Hormone	-	Increased
Glucagon	-	-
Cortisol	?	?

- Unchanged, ? changes unknown Source : Williamson (1980), Vernon (1988), Vernon (1989)

Insulin is the body's major anabolic hormone and in both rodents and ruminants, lactation is associated with hypoinsulinaemia (Vernon 1989, Williamson 1980). In rodents, this fall is thought to result from both a reduced glyceamic stimulus to the pancreas (Madon *et al.* 1990) and the destruction of insulin by the mammary gland (Jones *et al.* 1984). In addition to this reduced serum concentration, peripheral tissues (adipose tissue and muscles) become less responsive to insulin in both lactating rats (Burnol *et al.* 1986a, Burnol *et al.* 1987) and sheep (Vernon 1986, Vernon *et al.* 1990). In rodents however, there is evidence to suggest that lactation also results in an increased response to insulin by both the liver (Burnol *et al.* 1986b) and mammary gland (Burnol *et al.* 1987). Such changes therefore favour a reduced nutrient utilisation by peripheral tissues while promoting the anabolic processes in the liver (lipogenesis) (Williamson 1980) and mammary gland (lipid and protein synthesis) (Williamson 1986). Although the ruminant mammary gland is not receptive to insulin (Vernon *et al.* 1983), the problem of promoting mammary gland metabolism during lactation is resolved using an alternative mechanism that is described below. The hypoinsulinaemia during lactation is also beneficial to ruminants in allowing increased hepatic gluconeogenesis (Vernon 1988).

The importance of growth hormone for lactation in ruminants is now well established and its serum concentration rises considerably during early lactation (Vernon 1989). Although growth hormone does not act directly upon the mammary gland (Gertler *et al.* 1983) it stimulates the liver to produce an insulin-like growth factor (IGF-1) to which the mammary gland is responsive (Campbell *et al.* 1986). The increased serum IGF-1

concentration in ruminants has been proposed to have a similar role to that of insulin in rodents and thus promotes mammary gland metabolism. Growth hormone in lactating ruminants has also been shown to promote an increased hepatic glucose output *in vitro* (Pocius *et al.* 1986) and favours the partitioning of available nutrients (glucose, amino acids, lipid precursors) towards the mammary gland (Peel *et al.* 1981). Such an increase in growth hormone is not observed in rodents and more important to successful lactation is the rise in prolactin (Williamson 1980). Prolactin is involved in the development and differentiation of the mammary gland, and during lactation is released in response to the suckling stimulus. The magnitude of release reflects the suckling intensity in rats and has been suggested to indicate the mammary gland's continued requirement for nutrients (Bauman *et al.* 1983). However, although prolactin stimulates the metabolism and nutrient uptake (Vina *et al.* 1981) of mammary epithelial cells, whether prolactin acts as a homeorhetic regulator of other tissues during lactation is uncertain. Its action on the adipose tissue of rodents is thought to occur indirectly since this tissue does not possess prolactin receptors (Vernon *et al.* 1983).

In addition to the changes in serum hormone concentrations mentioned above, nutrient partitioning can be further supported by alterations in the response of tissues to hormones that are antagonistic to the actions of insulin. In lactating rodents, the mammary gland is not responsive to glucagon (Robson *et al.* 1984) while adipocyte sensitivity is increased *in vitro* (Zammit 1988). In ruminants, the change in the insulin:glucagon ratio during lactation may also have important implications for hepatic glucose production (Vernon 1988). Catecholamines, released by sympathetic nervous activity, may also be involved in nutrient partitioning as they actively stimulate lipid mobilisation. In lactating ruminants, tissue responsiveness to catecholamine action may be increased during lactation by the actions of other hormones (growth hormone) (McCutcheson *et al.* 1986, Vernon *et al.* 1985).

The concept that lactation is supported by the co-ordinated partitioning of available nutrients towards the mammary gland is therefore well established. Furthermore, it is also acknowledged that the mammary gland not only receives nutrients of dietary origin but the

nutrient supply can also be supplemented by the catabolism of endogenous reserves of fat, protein and minerals. However, despite this, there remains considerable variation in the extent to which the mechanisms involved in the partitioning of individual nutrients are understood. The control of lipid partitioning and mobilisation during lactation has received considerable attention in recent years and the processes involved have been described in detail for both farm livestock and laboratory animals. However, despite the importance of an adequate amino acid supply for milk production, few hard facts on the control of protein partitioning have been established. Information on the alterations in tissue protein turnover involved, the quantity of maternal protein available, the conditions under which mobilisation occurs and the quantitative importance of such adaptations remain to be elucidated. How the contributions of endogenous protein and energy yielding nutrients interact and influence lactational performance also remain uncertain. The current understanding of the processes associated with the partitioning of the major milk precursors is as follows.

LIPID PARTITIONING

The shift in metabolism associated with the partitioning of milk fat precursors during lactation involves alterations in both adipose tissue and the mammary gland. Adipose tissue, stores lipid as triglycerides in adipocytes whose functions include the synthesis, storage and mobilisation of lipid. In both ruminants and rodents, the onset of lactation is associated with reductions in adipocyte lipogenesis and the activity of key lipogenic enzymes (Grichting *et al.* 1977, Sinnet-Smith *et al.* 1980, Vernon *et al.* 1981). At the same time, the rate of triglyceride hydrolysis is increased (Smith *et al.* 1976, Zammit 1988) while the re-esterification of released fatty acids is also considerably reduced (Metz *et al.* 1977). During the same period, adipocyte lipoprotein lipase activity, the enzyme involved in fatty acid uptake from circulating lipoproteins, has also been shown to be significantly impaired in rats (Hamosh *et al.* 1970), cattle (Shirley *et al.* 1973) and sheep (Vernon *et al.* 1981), while the increased activity of this enzyme in the mammary gland facilitates the uptake of milk fat

precursors by mammary epithelial cells (Hamosh *et al.* 1970, Mendelson *et al.* 1977). Such changes in adipocyte metabolism therefore reduces its utilisation of lipogenic precursors (glucose, acetate and triglycerides) while actively enhancing the supply available to the mammary gland, where uptake and utilisation is considerably increased.

The above changes in adipocyte metabolism will be favoured by the hypoinsulinaemia and reduced adipocyte insulin sensitivity associated with lactation which may be mediated by serum prolactin (Ros *et al.* 1990). There is also some evidence to suggest that prolactin is responsible for the reciprocal changes in adipocyte and mammary gland lipoprotein lipase activity (Zinder *et al.* 1974) and lipogenesis (Ros *et al.* 1990). The hormonal environment also favours increased hepatic lipogenesis in rodents (Williamson 1980) and ruminants (Vernon 1988), while ketone body production is increased in ruminants as a fuel source for extra hepatic tissues. A decrease in non-shivering thermogenesis by brown adipose tissue in rodents further spares lipid precursors for lactation (Trayhurn *et al.* 1982).

During gestation, energy balance often becomes positive and a considerable amount of fat is stored in adipose tissue in preparation for lactation (Garnsworthy 1988, Naismith *et al.* 1982, Taggart 1961). The use of such fat reserves in support of lactation has been reported for dairy cows (Bauman *et al.* 1983), sheep (Cowan *et al.* 1979), humans (Butte *et al.* 1984), rabbits (Partridge *et al.* 1983) and rodents (Friggens 1990, Naismith *et al.* 1982). Although it might be suggested that lactation *per se* requires the use of body fat, the loss of adipose tissue is not obligatory and depends upon the size of the available reserves at parturition (Garnsworthy 1988, Rolls *et al.* 1984) and perhaps the animal's genetic potential for milk production (Oldham *et al.* 1989). At parturition in ruminants and rodents, changes in adipocyte metabolism promote the release of non esterified fatty acids (NEFA), although in guinea pigs this mobilisation is possibly initiated during the latter stages of gestation (Jones 1976). The loss of adipose tissue in rodents is also primarily associated with a reduction in cell size and not number (Steingrimsdottir *et al.* 1980).

For females well nourished during gestation, the proportion of body fat considered capable of being lost during lactation is likely to be very high and animals can become extremely thin provided membrane bound lipids are not disrupted. Previous studies have suggested that up to 60 % of body fat stores can be lost during lactation in ruminants (Vernon *et al.* 1984) and rodents (Naismith *et al.* 1982). Robinson (1986) recently described possible rates of body fat loss during pregnancy and lactation (Table 1.4) for a number of mammalian species. The possible maximum contribution of body fat to nutrient supply in the dairy cow has been estimated to range from 1 - 2.9 kg/d and at a peak fractional rate of 0.064/d (Konig *et al.* 1979, Vernon *et al.* 1984). For rodents however there appears to be more variability and, assuming a consistent rate of loss during lactation, calculated rates range from 0.027 d^{-1} (11.3 g/21d; Moore *et al.* 1984) to 0.046 d^{-1} (27.6 g/14d; Friggens 1990). Despite these estimated variations, the use of maternal body fat can provide a considerable contribution to a female's nutrient supply, with high yielding dairy cows during early lactation supplying over 54 % of milk fat carbon from body reserves (Wilson *et al.* 1988) while in rodents almost 25 % of the energy cost of lactation was provided by endogenous lipid (Naismith *et al.* 1982).

Table 1.4. Possible rates of body fat loss in different species during lactation.

SPECIES	FAT LOSS (g/d)
Mouse	0.044
Rat	1.54
Rabbit	7.50
Human	30
Pig	180
Sheep	200
Dairy Cow	1000

Source : Robinson (1986)

Body fat reserves can also have important and variable influences on lactational performance under conditions of nutritional adversity. Females that are fatter at parturition, and therefore have a greater capacity for body fat loss during lactation than their leaner contemporaries, can support a higher milk production when maternal feed intake is constrained either by its physical quality in ruminants (Jones *et al.* 1989) or by its limited

availability in pigs (Mullan *et al.* 1989a). Body fat mobilisation by lactating sows can also maintain milk quality and energy output under conditions of dietary energy restriction (Noblet *et al.* 1986). However, whilst it appears that a female's capacity for body fat mobilisation and maintenance of milk production are closely linked, the loss of body fat during lactation can also have significant metabolic consequences. The feed intake of fatter females appears to be constrained by this greater fat loss during lactation when compared to that of their leaner contemporaries offered the same high protein/high energy diet (Jones *et al.* 1989, Mullan *et al.* 1989a, Rolls *et al.* 1986). In lactating rodents, such body fat loss has catastrophic effects on the feed intake and hence milk production of females offered diets of low protein:energy ratio or low protein diets that have variable carbohydrate and fat contents (Friggens 1990, Naismith *et al.* 1982). In both studies, the situation was further exacerbated by the loss of body fat being unaffected by the dietary treatments. It has been proposed that a limiting heat loss capacity and ability to dispose of surplus energy yielding nutrients constrains intake (Friggens 1990), and prevents sufficient protein from being consumed thus limiting milk production. Therefore, while the partitioning of available milk fat precursors is central to a successful lactation, the balance between available nutrition and the endogenous fat supply can have significant metabolic consequences on subsequent lactational performance.

GLUCOSE PARTITIONING

Lactation imposes an enormous demand on a female's glucose supply since the mammary gland's requirement for lactose, glycerol and non-essential amino acid production is considerably increased. For lactating rodents, diets of limiting carbohydrate or gluconeogenic precursor content can significantly impair milk production (Friggens 1990, Koski *et al.* 1990), although on normal laboratory diets the carbohydrate content and increased intake prevent such insufficiencies from occurring (Williamson 1980). Lactating ruminants encounter an even greater problem of satisfying the gland's requirement, which can be 3 kg/d or more in a

high yielding dairy cow (Mepham 1987), since the bulk of dietary carbohydrate is fermented by rumen microbes to lipogenic end products.

Under conventional management practices, only a very small proportion of a lactating ruminant's glucose requirement can be met by glucose absorption from the digestive tract and it requires a two - threefold increase in hepatic gluconeogenesis to provide the bulk of the glucose supply (Vernon 1988). Although extra glucose can be provided from liver glycogen stores and renal gluconeogenesis, the contribution of these to the lactational glucose supply is minimal. The substrates utilised in gluconeogenesis include absorbed propionate and amino acids, glycerol released from adipocyte lipolysis and skeletal muscle amino acids (Bauman *et al.* 1983). This increased endogenous glucose production by lactating ruminants is favoured by the hypoinsulinaemia associated with lactation.

Despite this increased endogenous glucose production, the ruminant mammary gland at peak yield utilises up to 85 % of the available glucose supply (Bickerstaffe *et al.* 1974) leaving only 15 % for use by other tissues. In lactating rodents, mammary glucose uptake is also a considerable proportion of the total glucose supply and at peak yield approximates to 30 mmol/d (Williamson 1980). Maternal metabolism must therefore undergo alterations that partition available glucose towards the mammary gland. During lactation, glucose utilisation by peripheral tissues is considerably reduced in ruminants (Vernon 1988) and rodents (Williamson 1986) while muscles use fatty acids and ketone bodies as alternative energy sources. This glucose sparing by peripheral tissues is promoted by the hypoinsulinaemia and reduced tissue responsiveness associated with lactation (Burnol *et al.* 1987, Metcalf *et al.* 1990, Vernon *et al.* 1990).

MINERAL PARTITIONING

As might be expected, the functioning mammary gland also imposes an enormous demand on a female's mineral supply, particularly that of calcium, phosphorus, sodium and potassium. Despite the increased nutrient supply during lactation, the onset of milk secretion

is associated with considerable alterations in the partitioning of dietary and stored minerals (Bauman *et al.* 1983).

Studies of calcium and phosphorus metabolism during lactation in rodents have suggested that two major mechanisms are involved in mineral partitioning; the improved efficiency of intestinal absorption and the mobilisation of mineral from skeletal stores (Halloran *et al.* 1980a, Halloran *et al.* 1980b, Komorkova *et al.* 1969). Bone calcium loss during these studies was considerable and could represent between 23 - 38 % of total bone calcium, while the measurement of calcium and phosphorus fluxes during lactation have suggested that up 19 % of milk calcium can be derived from maternal skeletal stores (Brommage 1989). Such a loss of bone mineral in women has been suggested to be associated with increased levels of calcitonin and parathyroid hormone (Chan *et al.* 1987), although in that study the mobilisation of skeletal stores was prevented by adequate dietary calcium intakes. The intestinal absorption of calcium and phosphorus can also be increased during lactation, especially under conditions of dietary calcium inadequacy (Brommage 1989), and Bruns *et al.* (1987) concluded that this involved an increased production of the vitamin D dependent-calcium binding protein. The improved absorption of dietary calcium is stimulated by increased levels of the active form of vitamin D (1, 25$(OH)_2$ D_3) (Bruns *et al.* 1987) and it's synthesis is considerably increased during lactation in calcium restricted rats (Lobaugh *et al.* 1990).

Whilst these mechanisms are involved in the partitioning of calcium and phosphorus in lactating ruminants, at parturition and during early lactation they may be unable to maintain the calcium concentration in the blood and extra cellular fluid (ECF) and a condition known as parturient paresis can occur in dairy cattle. This condition can develop when either intestinal calcium absorption is reduced, following the possible feeding of a high dietary calcium:phosphorus ratio during pregnancy, or bone resorption is impaired through the interaction of a number of hormones including calcitonin (McDonald *et al.* 1981). The ability to mobilise bone mineral also decreases with age and in older cows it has been suggested that

bone resorption makes only a minor contribution to the total rate of calcium mobilisation and is of limited importance for the prevention of parturient hypocalcaemia (Van de Braak *et al.* 1987). Although this metabolic disease can be fatal, it can also be easily diagnosed and quickly reversed with an injection of a calcium solution (Webster 1987). The apparent effects of a reduced dietary cation-anion balance on plasma calcium concentration, its intestinal absorption and bone resorption have been suggested to be beneficial in the prevention of milk fever (Leclerc *et al.* 1989).

From the previous sections, it is apparent that a female's ability to adjust metabolism in a co-ordinated attempt to partition available nutrients towards the functioning mammary gland is central to successful milk production. Although the reciprocal changes in metabolic activity involved in the partitioning of lipid, glucose and available minerals are now well established, the rules governing amino acid utilisation remain to be elucidated.

PROTEIN PARTITIONING

Nutritionally, the supplies of glucose and amino acids represent the most critical factors that limit milk production, and since milk protein synthesis is a major function of mammary epithelial cells, the maintenance of amino acid supply is central to continued milk secretion.

In well nourished rodents and ruminants, whole body protein turnover undergoes considerable expansion during lactation as a result of increased protein synthesis in the mammary gland, liver and gastrointestinal tract (Baracos *et al.* 1991, Champredon *et al.* 1990, Millican *et al.* 1987) (Table 1.5). Such changes in protein metabolism make considerable demands on the available amino acid supply, and from Millican *et al.* (1987) protein synthesis in these tissues was calculated to have increased from 50 - 83 % of total body synthesis. In addition to amino acids being used in milk protein synthesis, they can also serve as gluconeogenic precursors, particularly important for ruminants, be used for tissue

protein synthesis or as an energy source via oxidative metabolism, and the partitioning of amino acids away from these competitive metabolic roles would favour their continued use in milk production. Recent evidence suggests that in lactating dairy cows essential amino acids are spared to some extent from oxidation (Black *et al.* 1990) while in rodents hepatic protein metabolism (urea synthesis) may be adjusted to spare amino acids for milk production (Barber *et al.* 1990, Naismith *et al.* 1987). However, the quantitative importance of these and other possible compensatory metabolic changes remain to be elucidated.

Table 1.5. Estimated rates of tissue protein synthesis in rodents and ruminants during lactation.

	LIVER		MAMMARY GLAND	
	FSR (%/d)	ASR (mg/d)	FSR (%/d)	ASR (mg/d)
Mice[1] : Pregnant	66	169	41	25
Lactating	73	439	150	799
Rats[2] : Lactating	66	1103	106	2075
Goats[3] :Dry	8.6	268*	2.8	12*
Lactating	9.1	339*	41.5	2242*

[1] Millican *et al.* (1987), [2] Sampson *et al.* (1986), [3] Champredon *et al.* (1990).
* ASR : mg/d/kg Empty Body Weight
FSR Fractional Synthesis Rate; ASR Absolute Synthesis Rate

In a similar way to that of milk fat precursors (triglycerides, NEFA), the amino acids utilised by mammary gland metabolism can be derived from two main sources, dietary protein following digestion and absorption and amino acids released from maternal protein stores. It is therefore of particular importance to the better understanding of protein partitioning that the possible controlling mechanisms involved in the partition of dietary and endogenous protein between sites of accretion (body) and secretion (mammary gland) are unravelled. Although the regulatory pathways of lipid partitioning have now been well established (Bauman *et al.* 1983, Vernon 1989), as yet few details are available for protein.

Although the partitioning of endogenous protein away from maternal protein stores has been reported to occur during lactation in a number of mammalian species, such a utilisation of protein reserves does not appear to be obligatory. It is therefore reasonable to

expect that factors that regulate the availability of maternal protein for catabolism during lactation ultimately determines the potential of such reserves to supplement dietary protein supply, buffer fluctuations in nutrient quality and availability, and thus support maternal milk production. Factors that are possibly instrumental in determining the availability of such reserves during lactation are:

1) The extent of protein reserve repletion.

2) Conditions associated with lactation.

3) The rate at which the available reserves can be depleted.

4) Controlling mechanisms of tissue protein metabolism.

The bulk of available information concerning the above statements is at present speculative and in need of some clarification. As a result of this lack of information, the quantitative importance of body protein reserves for milk production remains uncertain and is often estimated from assumptions of available reserves, milk yield and composition (Bauman *et al.* 1983).

Body Protein Reserve Repletion

Nutrients present in body tissues may be considered to constitute available reserves when they can be lost without deleterious effects on an animals functional integrity. It is now well recognised that tissue protein can provide such an available protein store not only in mammals but also in birds (Fisher *et al.* 1964, Sears *et al.* 1988) and plant seeds (Garcia-Augustin *et al.* 1989). From studies involving dogs and rodents, Allison *et al.* (1965) estimated that the available protein reserve can account for as much as 25 % of total body protein and concluded that such reserves are composed of those proteins that can be reversibly depleted/repleted and thus contribute to the body's free amino acid pool. Protein reserves in cattle, following a depletion/repletion experiment, have also been estimated to represent up to

25 % of body protein (Botts *et al.* 1979) and in a 600 kg dairy cow such reserves could provide up to 25 kg of protein (Mepham 1987). However, unlike adipose tissue whose main functions are the storage and mobilisation of lipid, available protein reserves are associated with functional proteins and not with any specialised storage organ. Despite this, the major contributions to the protein reserve are made by the body's musculature, particularly skeletal muscles, and skin (Allison *et al.* 1965, Swick *et al.* 1977) and it is thought that all muscle proteins are subject to such reversible degradation (Swick *et al.* 1977). From the studies of Allison *et al.* (1965) and Botts *et al.* (1979) it was also apparent that once the available protein reserves have been depleted, further loss of protein was in some way prevented and thus helped maintain the body's functional integrity. However, whilst this metabolic limit (25 %) to the available protein reserve may prevent excessive damage to the body's functional integrity after a short period of nutritional inadequacy, Allison *et al.* (1965) also reported that rats offered a protein free diet over longer periods could lose up to 50 % of body protein before succumbing to the rigours of such conditions.

Although the results of earlier studies have suggested that available protein reserves can account for up to 25 % of body protein in lactation, it must be noted that this possibly represents the extreme to which body protein can be used to supply amino acids for tissue metabolism, and in practical situations body protein content and hence protein reserves may have been previously depleted by various conditions. The quantity of protein available for mobilisation in support of lactation therefore depends on the extent of reserve repletion at parturition and is ultimately influenced by the prior demands on maternal protein supply and available nutrition both before and during gestation. Numerous studies involving rodents have concluded that under conditions of dietary protein and energy restriction, maternal protein reserves can be mobilised during gestation in support of the developing feto-placental unit (Anderson *et al.* 1980, Lederman et al. 1981, Moore *et al.* 1984, Zartarian *et al.* 1980). Furthermore, Naismith *et al.* (1976) have suggested that under normal circumstances gestational protein metabolism undergoes biphasic alterations, with protein being stored

during early gestation for catabolism during the latter stages in support of rapid foetal growth. It is therefore apparent that the extent of reserve repletion at parturition may not only be distinctly lower than the 25 % of body protein previously suggested, but, as a result of gestational changes in maternal protein mass, may not reflect the size of the available stores at the start of the reproductive cycle. Thus for many studies investigating the impact maternal protein reserves can have on sustaining milk production, consideration should be first given to possible variations in reserve repletion at parturition before appropriate conclusions are made.

Botts *et al.* (1979), in addition to estimating the possible size of available protein reserves, also reported that when severely protein depleted cows were offered a high protein ration (22 %) during lactation, they partitioned a larger proportion of the dietary protein supply towards the replenishment of their depleted protein stores than others offered a lower protein ration (18 %). This shift in protein partitioning, although promoting an increased rate of reserve repletion, significantly reduced milk yield when compared to cows offered the 18 % ration. Therefore the extent of maternal protein reserve repletion at parturition may not only influence the quantity of protein available for mobilisation, but may also adjust the partitioning of dietary protein between sites of accretion and secretion.

Conditions Associated with Lactation

Labile reserves of tissue protein obviously exist. Their mobilisation during lactation has been reported for a number of mammalian species, including dairy cows (Belyea *et al.* 1978), sheep (Lynch *et al.* 1988), goats (Baracos *et al.* 1991), pigs (Shields *et al.* 1985), rats (Friggens 1990, Naismith *et al.* 1982) and humans (Motil *et al.* 1989). Such a mobilisation of body protein is thought to be required to supplement an inadequate nutrient supply and the amino acids can be used to not only support milk protein synthesis but also gluconeogenesis, oxidative energy production and the maintenance of tissue protein integrity.

During early lactation in the dairy cow, appetite often increases more slowly than milk yield and a shortfall develops between nutrient supply and demand (Bauman *et al.* 1983).

It is during this period that the mobilisation of endogenous protein reserves is thought to occur and supplement the inadequate dietary supply. The results of a number of studies have supported this suggestion (Belyea *et al.* 1978, Wilson *et al.* 1988), while a recent investigation has estimated that more than 12 % of body protein was lost during the first two months of lactation (Gibb *et al.* 1992). Dairy cattle have also been shown to catabolise body protein when the protein content of the ration is limiting (Polan *et al.* 1985).

For lactating rodents, since peak milk yield occurs some two-thirds into lactation, such a shortfall between dietary protein supply and metabolic demand is not thought to occur in dams offered adequate nutrition (Williamson 1980). However, when maternal nutrient supply is limited by either reductions in dietary protein quantity and, as a consequence of a nutrient imbalance, feed intake (Friggens 1990, Naismith *et al.* 1982) or by restricted feed availability (Glore *et al.* 1985), milk secretion is significantly impaired. Under these conditions, such females attempt to sustain milk production by mobilising their available reserves of protein (Friggens 1990, Glore *et al.* 1985, Naismith *et al.* 1982), although milk output cannot be maintained at the level of comparable well fed females.

For both rodents and ruminants, the capacity of lactating females to catabolise their endogenous protein reserves under conditions of inadequate nutrient supply also appears to be influenced by their maturity or parity. An older female or one that has already experienced lactation may impart a higher priority to such a physiological state and therefore be more willing to lose body protein than a younger growing female, and the results of recent studies involving dairy cows during early lactation (Bruckental *et al.* 1989) and feed restricted rodents (Young *et al.* 1985) tend to support this suggestion. By younger females being less prepared to lose body protein, they effectively sacrifice their current milk production in favour of future growth, and although some protein is lost, the changes in muscle metabolism and structure do not prevent further development and growth (Glore *et al.* 1983).

For lactating rodents, dam maturity is also thought to influence muscle protein loss from females not considered to be subjected to nutritional inadequacy. Kanto *et al.* (1980)

have reported the loss of body protein from full fed and restricted multiparous females to be 10.0 and 12.9 g over 19 days respectively, while in studies involving virgin rats, body protein loss was only evident in those subjected to severe nutritional stress (Glore *et al.* 1985, Moore *et al.* 1984, Naismith *et al.* 1982, Naismith *et al.* 1987). In these same studies however, of perhaps more importance for body protein loss has been the lactational stress (litter size) imposed on such females. While no protein loss was reported for well fed females in studies where litter size was restricted to less than 10 pups (Glore *et al.* 1985, Millican *et al.* 1987, Moore *et al.* 1984, Naismith *et al.* 1982, Naismith *et al.* 1987), increasing the lactational stress to 12 pups, regardless of dam maturity, promoted muscle protein loss from both the control and treatment groups (Sainz *et al.* 1986a, 1986b, Taylor *et al.* 1986). These results suggest that when considering the total nutrient supply to lactating rodents, the loss of endogenous protein is not only determined by available nutrition but also the dam's own desire to utilise body protein and the size of the milk demand.

However, following a recent study (Friggens 1990) it might be concluded that for well nourished dams, the influence of dam maturity and litter size on body protein loss during lactation is limited. Using multiparous female rats suckling a large litter (13 pups), he reported that the feeding of a low protein/high carbohydrate diet impaired feed intake, and hence milk production, and necessitated the catabolism of body protein by such females (5.9 g/12d; 9 %) in an attempt to sustain milk output. Furthermore at this level of dietary protein, the replacement of dietary carbohydrate with fat promoted an even greater body protein loss (13.4 g/12d; 21%), although such a loss was unable to prevent litter growth from being compromised. As might have been expected, an increase in the protein content of the high carbohydrate diet provided a balance of nutrients that allowed feed intake and litter growth to increase throughout lactation, but without any loss of body protein. However, while reductions in the carbohydrate content of this high protein diet (replaced with fat) resulted in a constrained feed/protein intake, compromised litter growth (decreased protein, increased fat gain) and the probable use of increasing quantities of dietary protein for gluconeogenesis,

there was little evidence to suggest that, even under these conditions, the available protein reserves were catabolised in support of lactation. Thus these results might suggest that rats, at least, can tolerate a range of 'protein adequacy' for milk production and will only call upon protein reserves when amino acid supply is substantially inadequate. Under such conditions, the suckling stimulus and dam maturity are not particularly important in promoting body protein loss.

For dairy ruminants, the use of endogenous protein reserves during lactation may be influenced in a similar way to that previously reported for body fat (Neilson *et al.* 1983) by the animal's genetic potential for milk production. A recent study by Wilson *et al.* (1988) estimated that during early lactation, the proportion of milk protein and lactose derived from body protein was greater in high genetic merit dairy cows than in animals of a lower genetic potential.

Potential Rates of Maternal Protein Reserve Depletion

The potential of maternal protein to support milk production not only depends upon the mass of the available reserves but also the rate at which such protein can be released for use in maternal metabolism. Ultimately, the principle that a metabolic limit determines the extent to which maternal protein can become depleted, also applies to the rate of amino acid release from such protein reserves and will be determined by the balance between rates of muscle protein synthesis and degradation. However, at present information on possible rates of protein release is limited.

For the lactating dairy cow, Reid *et al.* (1966) estimated that during early lactation body protein loss could supply as much as 0.36 kg/d of protein in support of milk production. However, since in practical situations high yielding dairy cows do not lose body weight at more than 1 - 2 kg/d, Bauman *et al.* (1983) concluded that the maximal protein supply occurring *in vivo* is between 0.15 - 0.30 kg/d. Although this supply of endogenous protein may be critical during early lactation, it must be noted that such a rate of protein loss is

considerably less than the possible rates of lipid mobilisation (Konig *et al.* 1979, Vernon *et al.* 1984). The rate of muscle protein loss during lactation is unlikely to be fixed and may be adjusted in response to variations in nutrient supply and milk output, and Botts *et al.* (1979) reported that during a depletion/repletion experiment, the time required to deplete maternal protein reserves was dramatically reduced in cows with higher milk yields. From this observation, the authors suggested that rates of muscle protein loss were increased in such females, although whether this was promoted by the higher milk yield or whether the extra protein supplied increased milk production is unknown.

In non ruminants, available information on rates of maternal protein loss during lactation is also extremely limited and at present rates are calculated from changes in maternal protein mass during a fixed period of lactation. In such calculations of protein catabolism, it is normally assumed that muscle protein loss occurs at a consistent rate throughout the study period, although such an assumption may be grossly inaccurate under certain circumstances (see later). Calculated rates of maternal protein catabolism from a number of studies involving lactating rodents are shown in Table 1.6. The considerable variation in calculated rates that exists between studies may reflect differences in the nutritional and lactational stress imposed, the length of the study period used and possible differences in the source of the lost protein. Such differences may also indicate variations in the demand on maternal protein or the capacity of the females involved in each study to mobilise their endogenous reserves.

The extent of reserve repletion at parturition may also have an important influence on the absolute rate (g/d) of protein loss during lactation. The rates of loss calculated from Friggens (1990) and Sainz *et al.* (1986b) (Table 1.6), suggest that females entering lactation with comparable maternal protein reserves, can catabolise body protein at different rates in response to variations in nutritional adversity and can therefore support milk production to a greater or lesser extent. However, whether this ability applies to females that begin lactation with distinct differences in their mass of body protein is unknown. If a similar nutritional treatment is applied to such females, protein depleted mothers could lose what remains of their

protein reserves at a similar absolute rate but greater fractional rate (%/d) than the more replete females, although as a consequence the depleted females would reach their metabolic limit earlier. Alternatively, the fractional rate of loss may be similar in both groups but the contribution of body protein (g/d) to milk production by the depleted group would be considerably reduced. In spite of this, whatever rates of protein loss are established, once the metabolic limit of protein reserves are reached, further loss would be prevented in both groups (Glore *et al.* 1985). Such potential variation in the rate of protein loss deserves further attention.

Table 1.6. Calculated rates of body protein loss from lactating rats.

STUDY	DIETARY TREATMENT	PROTEIN LOSS (g)	RATE (g/d)
1	*Ad libitum*	10.0	0.52
1	Restricted (60 % *Adlib*)	12.9	0.68
2	Low protein/high CHO	5.8	0.48
3	Low protein/high CHO	5.9	0.49
3	Low protein/low CHO	13.4	1.12
4	Low protein/*adlib*	4.1	0.59
4	Low protein/restricted	8.3	1.19

CHO - Carbohydrate
Studies : 1, Kanto *et al.* (1980); 2, Naismith *et al.* (1982); 3, Friggens (1990); 4, Sainz *et al.* (1986b)

From changes in the litter growth of dams offered a low protein/low carbohydrate diet (Table 1.6), Friggens (1990) suggested that during lactation the protein reserves of such females were exhausted sometime before the end of the study period and resulted in a substantial fall in milk secretion. Therefore, while the assumption that body protein loss is linear throughout the study period allows rates of protein catabolism to be calculated, it ignores possible deviations from this pattern and may result in an underestimation of the maximum rate of protein loss occurring *in vivo*. If the contribution of maternal protein to milk production is to be better understood, possible variations in the rate of protein catabolism need to be clarified.

Controlling Mechanisms of Muscle Protein Metabolism

The shift in protein partitioning that promotes the release of amino acids from muscle protein reserves during lactation ultimately involves an alteration in tissue protein turnover such that degradation exceeds synthesis. Although it is now recognised that skeletal muscles represent the major site of possible mobilisation, the adaptive mechanisms involved have not been fully elucidated.

From numerous studies involving growing rats, the changes in muscle protein metabolism required to promote net protein breakdown are now well established. During starvation or the feeding of a protein free diet, both synthesis and degradation are initially decreased. However, prolonged exposure to such treatment results in protein release through an increase in degradation during starvation or a further fall in synthesis on the protein free diet such that degradation exceeds synthesis (Millward *et al.* 1978, Waterlow *et al.* 1978).

However, despite the contribution that muscle protein often makes to the available nutrient supply during lactation, such changes in muscle metabolism have not been fully established. From the limited number of studies that have investigated tissue protein metabolism in lactating ruminants, it might appear that changes in muscle protein turnover involved in net protein loss were species specific, since data from work involving lactating sheep suggested that an increase in degradation was primarily responsible (Vincent *et al.* 1985), while recent studies by Champredon *et al.* (1990) and Baracos *et al.* (1991) using dairy goats indicated that a fall in protein synthesis was involved, although in these latter studies rates of degradation were not determined. Other workers (Bryant *et al.* 1982) have suggested that both components of protein turnover are important, with the particular changes involved being dependent upon the individual muscle.

For lactating rodents, although separate studies have confirmed that muscle protein metabolism is not adjusted under conditions that do not promote body protein catabolism (Millican *et al.* 1987, Siebrits *et al.* 1985), such a loss of muscle protein from dams subjected to severe protein restriction could not be attributed to any change in carcass protein turnover

(Sainz *et al.* 1986b), although the authors suggest that the aggregation of many protein pools could have masked the measurement of possible changes. Alternatively, from the use of indirect measurements of muscle metabolism, DeSantiago *et al.* (1991) suggested that changes in both protein synthesis and degradation were responsible for net muscle protein loss, while Sainz *et al.* (1984), using the excretion of 3-methylhistidine, concluded that an increase in protein degradation was involved, although uncertainties over the source of this metabolite in urine limits its validity as an index of muscle protein degradation (Harris *et al.* 1977, Wassner *et al.* 1982). From these studies it is apparent that not only do the changes in muscle protein turnover that regulate net protein loss during lactation remain to be clarified, but also the quantitative changes in such metabolism that allow possible variations in the rate of loss and prevent the excessive catabolism of muscle protein beyond the metabolic limit.

In addition to the changes in tissue protein metabolism that are involved in the shift of protein partitioning, associated support processes may also undergo some alteration in activity. Many amino acids are transported into muscles via Na^+ dependent mechanisms and there is evidence to suggest a close link between the activity of the enzyme Na^+,K^+-ATPase (EC 3.6.1.3) and muscle protein synthesis (Adeola *et al.* 1989, Vandenburgh *et al.* 1981). Changes in the activity of this enzyme and associated rates of protein synthesis may provide an additional control point for protein partitioning during lactation, although this, as yet, has not been established.

Although in both ruminants and non ruminants the hypoinsulinaemia (Vernon 1988, Williamson 1980) and reduced skeletal muscle insulin responsiveness associated with lactation (Burnol *et al.* 1987, Metcalf *et al.* 1990, Vernon *et al.* 1990) will limit the anabolic effects of insulin on muscle protein metabolism (stimulating amino acid uptake and protein synthesis, inhibiting degradation) (Waterlow *et al.* 1978), and favour the partitioning of nutrients (glucose, amino acids) away from these tissues, the metabolic signal(s) that promotes the increase in muscle protein breakdown is unknown. Whilst a change in the nutrient supply to muscle is not thought to have a direct effect on the control of muscle protein

turnover via a mass action mechanism (Reeds *et al.* 1983), such changes in nutrient supply may influence protein turnover indirectly through alterations in the animal's hormonal status.

One hormone thought to be associated with muscle protein catabolism in protein restricted growing rats is cortisol (Millward *et al.* 1983), and Naismith (1966) has postulated a similar role for the increasing levels of circulating corticosteroids (Voogt *et al.* 1969) in promoting muscle protein loss during the latter stages of gestation. Whilst the catabolic effects of corticosteroids on muscle protein mass have been shown to involve a reduction in protein synthesis (Waterlow *et al.* 1978), other evidence suggests that it may also be associated, to a lesser extent, with an increase in degradation (Odedra *et al.* 1982) and the efflux of amino acids from muscle cells (Lewis *et al.* 1982, Rannels *et al.* 1980). Since insulin and cortisol are thought to have opposing effects on muscle protein metabolism (Southorn *et al.* 1990), possibly of more importance than their individual concentration will be their circulating ratio (Buttery 1983), and it has been suggested that an increase in the corticosterone/insulin ratio would favour the loss of muscle protein in female rats offered diets of poor quality protein during lactation (Grimble 1981). While it remains to be established that this balance of hormones is primarily responsible for the control of protein loss during lactation, a recent study suggested that serum cortisol levels were considerably increased in protein restricted lactating rats (Kliewer *et al.* 1987). Changes in the activity of the thyroid gland may also have a role in promoting muscle protein release during lactation since hyper-thyroidism is also associated with increased muscle protein catabolism (Buttery 1983) and stimulate muscle proteinase enzymes (DeMartino *et al.* 1978), although the effect of physiological changes in thyroid hormone levels in protein deficient rats are difficult to interpret (Jepson *et al.* 1988). The possible involvement of other metabolites that regulate muscle protein synthesis and degradation, such as prostaglandins (Goldberg *et al.* 1984, Rodemann *et al.* 1982) and branched chain amino acids (Goldberg *et al.* 1978), in the promotion of maternal protein mobilisation during lactation is at present uncertain and would merit some research attention.

Muscle protein breakdown ultimately depends on the activity of tissue proteolytic enzymes, and Goll *et al.* (1983) classified the enzymes involved in the maintenance of muscle integrity into three groups, lysosomal hydrolases (cathepsins), neutral and alkaline proteases, and the calcium dependent proteinase (calpain). Rather than working in isolation, muscle protein breakdown involves the action of a number of enzymes, and Mortimore *et al.* (1987) recently concluded that myofibrillar protein breakdown requires the initial activity of the calcium activated proteinase before other enzymes can become involved. To prevent unnecessary and damaging protein catabolism, the activity of these muscle proteases needs to be closely regulated. This is aided by the packaging of enzymes into lysosymes, and that the calcium activated proteinase exists in an active/inactive form that can be closely regulated by an intracellular inhibitor and cellular conditions (Higgins *et al.* 1988). Although the activity of these muscle proteases are obviously closely regulated, little information is available at present on the activity of such enzymes in the mobilisation of maternal protein reserves during lactation and how such protein degradation is regulated to prevent excessive catabolism beyond the metabolic limit. Furthermore, whilst Swick *et al.* (1977) have proposed that body protein reserves are not composed of any specific storage polypeptide, the evidence that suggests muscle protein fractions respond differently to dietary (Rikimaru *et al.* 1980) and anabolic stimuli (Adeola *et al.* 1992) might indicate the possibility of comparable changes during lactation.

Although it is now accepted that, in a similar way to adipose tissue acting as a store of fat, body tissues (muscles) can also act as a store of protein which can be drawn upon during times of nutritional adversity or increased demand (lactation), the previous sections clearly show that the information available on the use of such reserves during lactation is limited. The importance of maternal protein stores in sustaining milk production under various conditions is therefore difficult to quantify.

For the lactating diary cow, Bauman *et al.* (1983) estimated that body protein stores could contribute to the production of 800 kg of milk during lactation, whilst on a daily basis

this endogenous protein supply could provide enough amino acids for up to 10 kg of milk. Although such estimations can provide a useful indication of the capacity of body protein to support milk production, they can only be made using assumptions of reserve repletion, maximal rates of protein loss and milk composition, and may not reflect the changes in body condition that are desired under conventional management conditions.

Since the potential value of a lactating female's body protein stores are constrained by metabolic limits to both their size and rate of loss, it is apparent that during periods of nutritional inadequacy such reserves cannot support milk secretion to the level of well fed females (Naismith *et al.* 1982, Polan *et al.* 1985). Despite this, several authors have concluded that the extent of body reserves at parturition can have a significant impact on the ability of lactating sows (Klaver *et al.* 1981), sheep (Peart *et al.* 1970) and rats (Kliewer *et al.* 1987) to maintain milk output when nutrient supply is limiting. However, in such studies no distinction was made between the body's protein or fat reserves and it is therefore difficult to assess the impact that the repletion of protein reserves had on milk production.

One study in which an attempt was made to directly manipulate the magnitude of tissue protein reserves at parturition and then investigate the subsequent effects of such variations on lactational performance was reported by Mahan *et al.* (1975) using lactating sows. Although measurements of body composition were not made, the authors concluded that when dietary protein was limiting during lactation, piglet growth could be almost 20 % greater in sows with a "Full" protein reserve as compared to their "Depleted" contemporaries. However, while a subsequent study from the same laboratory (Shields *et al.* 1985) confirmed that the depletion of maternal protein reserves during gestation ultimately limited the quantity available for catabolism during lactation and that such a limitation impaired the lactational performance of protein restricted sows, the impact of such variations on the maintenance of milk secretion are difficult to interpret because of the possible impact of severe protein restriction throughout gestation on mammary gland integrity and functional capability (Rosso

et al. 1981) or the effects of increased piglet mortality on litter size and thus lactational demand.

THESIS OBJECTIVES

From the above discussion it is apparent that our understanding of how lactating females partition their available protein supply and thus support the mammary gland's increased amino acid demand, especially during periods of protein undernutrition, is limited by the lack of information concerning the capacity of their endogenous protein reserves to support milk production and the changes in tissue metabolism associated with such utilisation. It was therefore decided to address this gap in our present knowledge and the objective of this thesis has been to improve our understanding of the following aspects of protein partitioning during lactation:

1) Factors that influence the utilisation of maternal protein reserves during lactation.: The loss of body protein during lactation may not only be influenced by nutrient supply, but also dam maturity and litter demand.

2) The effect of variations in the extent of reserve repletion at parturition on subsequent lactational performance when dietary protein is limiting: Whilst the use of body protein reserves during lactation is now widely established, the importance of the extent of reserve repletion at parturition on the capacity to sustain milk production remains to be established.

3) Whether the rate of maternal protein mobilisation can be varied: Alterations in the endogenous protein supply may be promoted by variations in nutrient supply and will ultimately influence the degree to which milk production can be maintained.

4) The controlling mechanisms of muscle protein metabolism that promote the loss of body protein during lactation: What are the changes in muscle protein turnover (synthesis/degradation) that are involved in both the promotion of muscle protein loss and the prevention of excessive catabolism beyond the proposed metabolic limit.

To investigate these topics, four experiments using rats as a model were carried out and are described in chronological order in the following chapters. Rats were chosen as the experimental animal because of the degree to which they can be manipulated, the ease of whole body analysis, the availability of proven methods for the analysis of tissue protein metabolism and the timescale of their reproductive cycle allows for a rapid accumulation of information.

CHAPTER TWO

EXPERIMENT E1

MATERNAL PROTEIN RESERVES and THEIR INFLUENCE on LACTATIONAL PERFORMANCE in RATS.

ABSTRACT

To determine the contribution of tissue protein reserves to lactational performance multiparous female Sprague-Dawley rats were mated, caged individually and offered a diet high in protein (H 215gCP/kg DM) *ad libitum* until day 12 of gestation. Subsequently half continued to receive diet H while the remainder were offered a diet low in protein (L 65gCP/kg DM) until parturition. This treatment aimed to produce a difference in carcass protein at parturition. On day 1 of lactation females were allocated to either diet H or a low protein diet (L_2 90gCP/kg DM) offered until day 13 of lactation, giving four lactation treatment groups HH, HL_2, LH and LL_2. Groups of females were slaughtered on days 2 and 12 of gestation and days 1 and 13 of lactation and carcass and major organs analysed. Weight gain of standardised litters was used as an indicator of lactational performance. Maternal carcass protein contents at parturition were 43.5 (± 1.2)g and 38.7 (± 0.8)g ($P<0.01$) for H and L respectively. During lactation there was little change in carcass protein content of HH while LH appeared to replenish their depleted reserves. Food Intake or lactational performance did not differ between these two groups. HL_2 and LL_2 lost carcass protein with HL_2 losing more than LL_2 ($P<0.05$). Intake and lactational performance were reduced compared to that on diet H ($P<0.05$) but for the first six days of lactation were both greater ($P<0.05$) for HL_2 than for LL_2. All four groups showed a considerable loss of body fat during lactation which was not affected by diet. The ability of HL_2 to catabolise more protein and consume more food allowed them to sustain a greater lactational performance. Previous maternal protein depletion had no influence on lactational performance as long as an adequate supply of dietary protein was provided.

The concept of mammals having a store of protein in tissues that is capable of being depleted in times of stress and thereby contributing to the free amino acid pools of the body has been well documented for rats (Allison *et al.* 1965), chicks (Fisher *et al.* 1964) and cattle (Biddle *et al.* 1975, Paquay *et al.* 1972). The body's major protein reserve is reported to be found in skeletal muscle (Swick *et al.* 1977) and can represent approximately 25 % of body protein (Allison *et al.* 1965, Botts *et al.* 1979).

Lactation imposes an enormous demand on a mother's protein and energy supply and although there is a concomitant elevation of food intake, the use of body fat stored during gestation has been shown to make an important contribution to the additional energy cost of lactation in rats (Naismith *et al.* 1982), humans (Butte *et al.* 1984) and cattle (Bauman et al. 1983) especially during early lactation. Maternal protein reserves may also be catabolised to supply amino acids for milk protein synthesis and gluconeogenesis in rats (Friggens 1990, Naismith *et al.* 1982, Sainz *et al.* 1986a), humans (Motil *et al.* 1989) and cattle (Belyea *et al.* 1978, Trigg *et al.* 1981, Wilson *et al.* 1988).

Although it has been well established that the degree of fatness at parturition influences subsequent responses to nutrition during lactation (Garnsworthy 1988), the situation regarding the influence tissue protein repletion has on lactation remains uncertain.

The utilisation of tissue protein reserves during lactation has been suggested to be important in allowing lactating sows to sustain lactational performance under conditions of dietary protein inadequacy (Mahan *et al.* 1975). The extent to which these reserves could influence lactational performance was also thought to depend on the size of the available protein reserve at parturition, although no quantitative measurements of tissue protein reserves were made. Friggens (1990) has also suggested that in rats the ability to sustain lactation on marginal diets depends upon the extent of maternal tissue reserves and the capacity of females to mobilise such reserves. Therefore in many studies of tissue mobilisation during lactation using rats as a model, the unstated assumptions that either the degree of repletion of tissue

reserves at parturition is not important to subsequent changes during lactation or that the reserves are fully replete (Naismith *et al.* 1982, Sainz *et al.* 1986a) are clearly not tenable.

The objective of the current study was to investigate the influence that the maternal protein reserves and the extent of their depletion have on lactational performance in rats offered an adequate or in-adequate dietary protein supply.

MATERIALS and METHODS

The current experimental protocol was designed to establish at parturition two groups of female rats that had distinct differences in the size of their maternal protein mass and thus protein reserve, which will be described as either "Full" or "Depleted". In this experiment (E1) only 4 (treatment H) and 3 (treatment L) rats were slaughtered at parturition for body composition analysis. This analysis identified maternal protein masses of 44.6 (± 1.7)g and 39.3 (± 1.7)g for H and L respectively which, by convention, were not significantly different (P=0.08). As the purpose of this work was to examine the impact of differences in maternal protein mass on lactational performance, it was felt necessary to consolidate these measurements of initial protein mass by amalgamating them with those from a complimentary experiment (E2) in which rats of the same type and from the same source were treated similarly during gestation and culled immediately after parturition on day 1 of lactation. This second study involved the same experimental protocol but was designed to investigate changes in tissue protein metabolism during lactation. Dams used in this complimentary experiment (E2) and offered treatments H and L during gestation had initial body weights of 300.7 (± 2.7)g and 303.9 (± 3.4)g respectively. Following parturition, the body weight of these females were 312.7 (± 4.0)g and 307.2 (± 5.8)g, whilst the carcass composition analysis of dams culled on day 1 of lactation established their body protein masses to be 42.3 (± 1.59)g and 38.6 (± 0.9)g for H and L respectively. Between the two experiments there was no significant difference in the relationship between carcass protein content and maternal body weight (from regression analysis). The experimental protocol is described in detail below.

Experimental Design

Multiparous female Sprague-Dawley rats (Harlan and Olac UK Ltd) were caged individually in a room regulated at 22 °C and humidity from 40 - 60 % with a light period from 08.00 - 20.00 hours. At the appropriate time, females were placed individually in a wire bottomed cage with a proven male breeder. The morning on which mating was confirmed, through the presence of vaginal plugs, was designated day one of gestation and the females were returned to solid bottom plastic cages for the remainder of the experiment.

Following mating the females were offered a high protein diet (H, 215 g CP/kg DM) (Table 2.1) *ad libitum* until day 12 of gestation. Subsequently half of the females continued to receive the high protein diet while the remainder were offered a low protein diet (L, 65 g CP/kg DM) *ad libitum* until parturition. Groups of females in experiment 1 (n=4) were selected at random for slaughter on days 2 and 12 of gestation and immediately following parturition. Their carcasses were analysed for dry matter, protein, ash and fat (see below). Litters from females slaughtered following parturition were also used for carcass analysis.

Table 2.1. Diet formulation (g/kg DM).

	HIGH (H)	LOW (L)	LOW (L_2)
Casein[1]	215	65	90
Corn Oil	191	236	229
Starch/Sucrose[2]	444	549	531
Vitamin Mix[3]	50	50	50
Mineral Mix[3]	100	100	100

[1] Casein supplemented with DL-Methionine (99% + 1%)
[2] Starch and Sucrose mixture in ratio 2 : 1
[3] Vitamin and Mineral Mix Formulated to meet N.R.C. requirements 1978
Diet Analysis : Protein (g CP/kg DM) H 214.8, L 67.7, L_2 90.9
GE (MJ/kg DM) H 21.3, L 21.2, L_2 21.4
Emulsifier (Lecithin) : 0.2% Fresh Matter
Antioxidant (Butylated hydroxy toluene) : 0.001% Fresh Matter

Dietary treatments described here for lactation relate to Experiment 1 animals only. On day 1 of lactation females were allocated factorially to either the high (H) or a low protein diet (L_2, 90 g CP/kg DM) which were offered *ad libitum* for the rest of the experiment. This allocation produced four groups of females (HH, HL_2, LH, and LL_2; the first letter

representing dietary treatment from day 12 of gestation and the second letter representing the lactation diet) that reached day 13 of lactation, at which point females and litters were slaughtered and analysed (see below).

All diets were formulated to provide 21 MJ GE/kg DM with a constant carbohydrate:fat ratio of 2.3:1. Litters were standardised to twelve pups on day 1 of lactation and litter weights were measured daily. Dam body weights and feed intakes were recorded daily throughout the experiment. All females were given free access to drinking water.

Carcass Analysis

Dams were killed by decapitation and the liver, mammary gland, gastrointestinal tract (empty), viscera and carcass were dissected from all animals and analysed for dry matter, protein, ash and fat (Appendix 1). The dry matter content was designated as the constant weight achieved following freeze drying. Protein was calculated as Kjeldahl N x 6.25. Ash content was estimated following combustion at 550 °C for 24 hours. Fat was estimated from the GE/kg DM of each carcass using the equation:

Carcass Fat (g/Kg DM)= (GE - 23.6 x Crude Protein/1000)/ 39.6/1000

where 23.6 and 39.6 represent the gross energy contents (MJ/kg) of crude protein and fat respectively (McDonald *et al.* 1981). Gross energy was estimated using a Gallenkamp bomb calorimeter.

The carcass composition of females slaughtered on day 13 of lactation was estimated for day 2 and 12 of gestation and day 1 of lactation by regression on body weight and composition of females offered similar dietary treatments slaughtered at each point. Regression equations produced for day 1 of lactation utilised data for females slaughtered in the two parallel experiments.

Statistical Analysis

For the statistical treatment of results two-way analysis of variance and one way analysis of variance were used, and where appropriate by the calculation of least significant differences, T-tests were used to compare between means of the four lactation treatment groups.

RESULTS

Feed Intake and Body Weight Changes During Gestation

The mean initial body-weights of the four treatment groups HH, HL_2, LH and LL_2 on day 1 of gestation were 321.3, 316.3, 325.7 and 322.3 (SD 5.1)g respectively. Feeding of the low protein diet during the second half of gestation reduced the body weight gains of pregnant females compared to those receiving the high protein diet ($P<0.001$) although there was no significant difference in feed intakes (Table 2.2).

Table 2.2. Maternal body weight gain, feed intakes and pup birth weight of rats offered a high (H) or low (L) protein diet from day 12 of gestation until parturition.

DIETARY TREATMENT	H n=14	L n=12	SD
Dam weight gain[1] (g) day 1-22	39.6	13.9	19.5^{***}
Feed intake (g DM) day 1-11	185.7	192.6	20.5^{NS}
Feed intake (g DM) day 12-22	204.4	185.3	29.8^{NS}
Litter size (pups/litter)	14.5	12.7	2.7^{NS}
Mean pup birth weight (g)	6.4	5.6	0.7^{**}

[1] Dam Weight Gain Following Parturition
DM Dry Matter
NS Non Significant : * $P<0.05$, ** $P<0.01$, *** $P<0.001$

The low protein treatment from day 12 of gestation had no significant effect on litter size but did significantly reduce the mean pup birth weight ($P<0.01$).

Effects of Gestational and Lactational Dietary Treatments on Dam Feed Intakes and Body Weight Changes During Lactation

The lactation results for the four treatment groups HH, LH, HL_2 and LL_2 are shown in Table 2.3. Body weight changes during lactation were significantly affected by dietary treatment during both gestation and lactation. Feeding of the low protein diet resulted in significantly more weight loss than occurred with the high protein diet. However the weight loss of group LL_2 was significantly less than that of group HL_2. Although the difference in lactational weight change between groups HH and LH was not significant, the 10 g gain by group LH might represent some degree of replenishment of maternal tissue reserves.

Table 2.3. Feed intakes, body weight losses and carcass composition changes during lactation of rats offered either diets H or L during gestation and then H or L_2 during lactation.

	DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION							
	HH n=6	LH n=4	HL_2 n=4	LL_2 n=5	SD	DIET EFFECT GEST.	LACT.	INTER.
Feed Intake (gDM/12d)	391.6	390.6	164.4	129.6	131.2	-	***	-
Day 1-6 (gDM)	142.9	140.7	88.7	45.3	46.4	*	***	*
Day 7-13 (gDM)	248.7	249.9	75.7	84.3	89.8	-	***	-
Dam Wt Change (g/12d)	-11.6	10.1	-109.1	-85.7	52.2	*	***	-
Dam Gains (g) of :								
Carcass Protein[a]	-1.6	1.9	-10.3	-5.8	5.2	*	***	-
Carcass Fat[a]	-15.5	-16.4	-20.5	-18.9	5.4	-	-	-

[a] Dam carcass composition changes adjusted for initial composition on day 1 of lactation using regression equations derived from data for females slaughtered in this experiment and experiment 2 and shown below :

Protein H = 20.9 + 0.0692 Body Weight (n=8, r^2=54.5%, P<0.05)
L = 18.2 + 0.0677 Body Weight (n=7, r^2=77.0%, P<0.05)
Fat - H = -39.8 + 0.192 Body Weight (n=8, r^2=85.5%, P<0.01)
L = -16.3 + 0.131 Body Weight (n=7, r^2=86.3%, P<0.01)

- not significant, * P<0.05, ** P<0.01, *** P<0.001

Gestational treatment had no significant effect on feed intakes of females offered the high protein diet during lactation and for groups HH and LH feed intakes rose throughout the period of lactation. However feeding of the low protein diet during lactation resulted in a

significant suppression of food intake when compared to that of the high protein groups. This suppression of intake was greatest for group LL_2, especially during the first 6 days of lactation ($P<0.05$). There was no significant difference in intake between groups HL_2 and LL_2 during the second half of lactation.

Effects of Dietary Treatment on Maternal Body Composition

Carcass Protein: Maternal protein rose from 49.6 (± 2.8)g to 53.1 (± 2.2)g between day 2 and 12 of gestation. Thereafter dams that continued to receive the high protein diet (H) reduced their body protein to 43.5 (± 1.2)g by day 1 of lactation. This reduction was significantly greater in those females offered diet L ($P<0.01$) and body protein was reduced to 38.7 (± 0.8)g. This confirms that the low protein gestation treatment was of sufficient severity to significantly deplete protein reserves in such females.

Subsequent changes in carcass protein content during lactation were not only related to the lactation diet offered but also to the initial state of carcass protein reserves (Table 2.3), as estimated from regression on live weight (see methods section). Groups HL_2 and LL_2 catabolised considerably more carcass protein between day 1 and 13 of lactation than either of the high protein groups, although the loss by group LL_2 was not significantly different to group HH ($P=0.07$). Groups HL_2 and LL_2 reduced their carcass protein contents to 35.2 (± 0.8)g and 35.3 (± 1.6)g respectively, while during the same period group HH maintained their carcass protein content and LH increased theirs from 40.3 (± 1.6)g to 42.6 (± 2.2)g. Changes in the carcass protein content of the four lactation treatment groups are shown in Fig. 2.1.

Carcass Fat: In all animals there was considerable storage of fat in the carcass during gestation between day 12 and parturition (Fig. 2.2). This accumulation of adipose stores was not affected by diet offered during the second half of pregnancy, and the carcass fat contents at parturition were 22.8 (± 2.5)g and 23.5 (± 1.5)g for dams receiving the H or L diets respectively. During lactation the four treatment groups all showed a considerable loss

of carcass fat; this loss was not significantly affected by gestational or lactational dietary treatment (Table 2.3, Fig. 2.2).

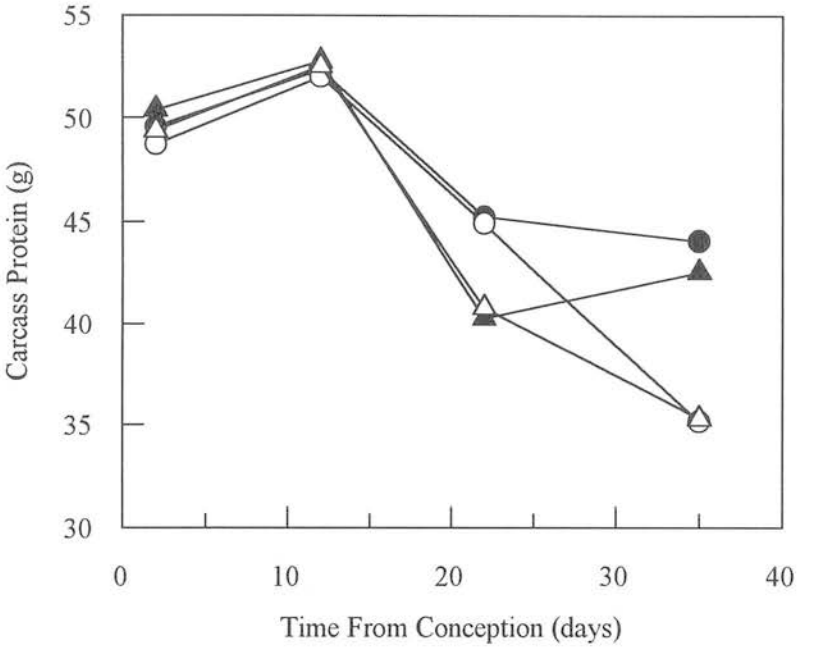

Fig. 2.1. Changes in the carcass protein content from day 2 of gestation to day 13 of lactation in groups of female rats offered either a high or low protein diet (H or L) from day 12 of gestation followed by either a high or low protein diet (H or L_2) during lactation, HH (●) (n=6), HL_2 (○) (n=4), LH (▲) (n=4) and LL_2 (△) (n=5). See text for details of dietary treatments.

There was also considerable accumulation of fat in the abdominal stores during gestation, rising from 11.9 (± 0.6)g on day 1 of gestation to 19.6 (± 2.3)g and 21.1 (± 2.3)g for dams receiving H or L diets respectively and culled on day 1 of lactation. The estimated loss of fat from the abdominal stores during lactation by the four treatment groups was also not significantly affected by gestation or lactation dietary treatment being 12.8, 16.3, 15.9 and 20.2 g for HH, LH, HL_2 and LL_2 respectively.

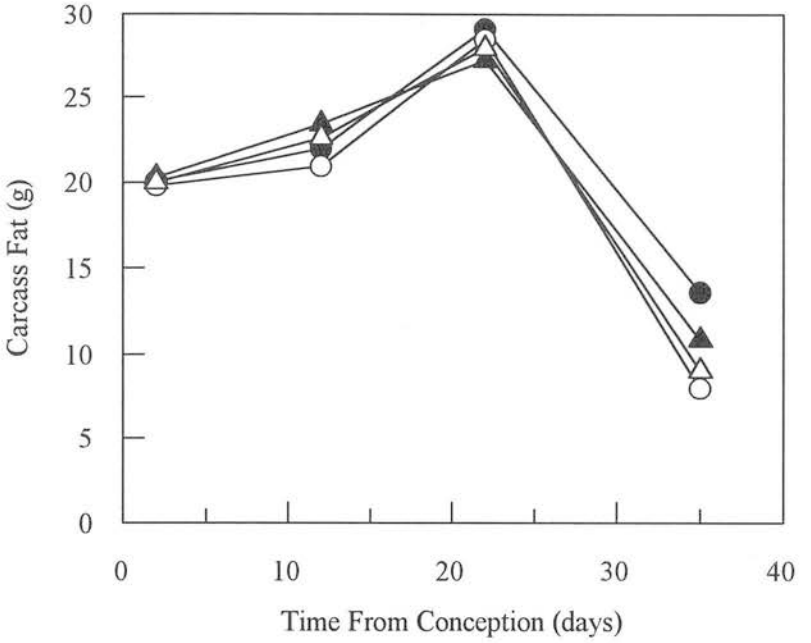

Fig. 2.2. Changes in the carcass fat content from day 2 of gestation to day 13 of lactation in groups of female rats offered either a high or low protein diet (H or L) from day 12 of gestation followed by either a high or low protein diet during lactation (H or L_2), HH (●) (n=6), HL_2 (○) (n=4), LH (▲) (n=4) and LL_2 (△) (n=5). See text for details of dietary treatments.

Effect of the Gestational and Lactational Dietary Treatments on Litter Weight Gain and Composition Changes During Lactation

Lactational performance, as represented by litter weight gain, was significantly greater in females that received the high protein diet during lactation ($P<0.001$) compared to that of the low protein groups, and in general followed the pattern shown for maternal dietary intakes (Table 2.3, 2.4). Gestation treatment had no significant effect on lactational performance of the high protein groups and litter weight gain continued to increase through lactation (Fig. 2.3). Although lactational performance was significantly impaired by the feeding of a low protein diet, the capacity of group HL_2 to mobilise greater quantities of carcass protein and consume more food than group LL_2 allowed them to maintain a significantly ($P<0.05$) higher lactational performance during the first six days of lactation than LL_2. However group HL_2 were unable to maintain this increased performance during the second half of lactation, at which time their litter weight gain was less than 50 % of that in the

first half. Group LL_2 showed very little difference in performance between the first and second half of lactation even though their food intakes almost doubled.

Table 2.4. The effect of gestational and lactational dietary treatments on litter weight gains and changes in litter composition during lactation.

	DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION							
	HH n=6	LH n=4	HL_2 n=4	LL_2 n=5	SD	DIET EFFECT GEST.	LACT.	INTER.
Weight Gain (g/12d)	264.9	270.9	87.5	61.1	102.4	-	***	-
Days 1 - 6	97.8	104.3	62.0	34.3	32.0	-	***	*
Days 7 - 13	167.1	166.6	25.5	26.8	73.3	-	***	-
Gains of (g/12d) :								
Protein[a]	38.7	37.9	14.6	10.0	13.9	-	***	-
Fat[a]	40.5	42.6	12.0	7.3	17.7	-	***	-

- not significant, * P<0.05, ** P<0.01, *** P<0.001

[a] Litter Protein and Fat Gains Adjusted for Initial Composition on day 1 of lactation

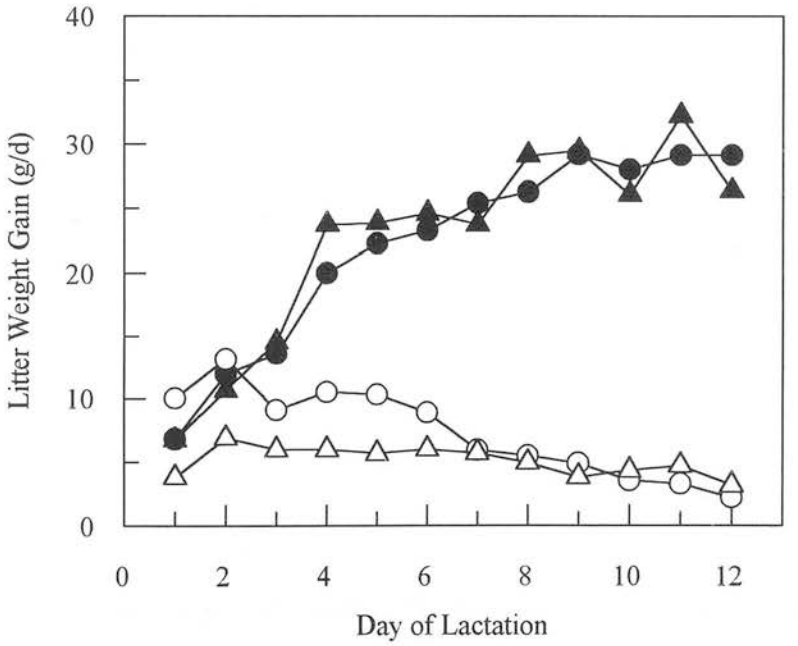

Fig. 2.3. Daily litter weight gain (g) during lactation of females offered either a high or low protein diet from day 12 of gestation (H or L) followed by either a high or low protein diet during lactation (H or L_2), HH (●) (n=6), HL_2 (○) (n=4), LH (▲) (n=4) and LL_2 (△) (n=5). See text for details of dietary treatment.

The significantly greater litter weight gain during lactation supported by the high protein diet was also reflected in a significantly greater litter gain in protein and fat compared

to the two low protein groups (Table 2.4). There was no significant difference in protein or fat gain between the two high protein groups. The greater lactational performance shown by HL_2 during lactation (significant during day 1 - 6) compared to LL_2 is reflected in the significantly greater litter protein gain, although there was no significant difference in their fat gains (Table 2.4).

Effect of Gestational Dietary Treatment on Maternal Organ Weight and Composition on Day 1 of Lactation

The maternal organ weights of females killed on day 1 of lactation are shown in Table 2.5. The low protein dietary treatment (L) from day 12 of gestation had generally no significant effect on either the weights of the major organs (liver, mammary gland and gastrointestinal tract) or their composition on day 1 of lactation. Only liver protein content was significantly reduced by the low protein treatment during the second half of gestation.

Effect of Gestational and Lactational Dietary Treatments on Maternal Organ Weight and Composition During Lactation

The maternal organ weights and compositions on day 13 of lactation for the four treatment groups HH, LH, HL_2 and LL_2 are shown in Table 2.6. Lactational dietary treatment, but not the gestational treatment, had a significant effect ($P<0.001$) on all measures reported in Table 2.6.

The feeding of the high protein diet during lactation increased the size and composition of the major organs analysed by day 13 of lactation when compared with sizes following parturition (Table 2.5). Both the liver and gastrointestinal tracts showed considerable increases in their weights (wet and dry) and protein contents, while liver fat content increased as G.I. tract fat declined. The mammary gland did not increase in size to the same extent as the liver and G.I. tract but it did show considerable changes in composition. As milk production had increased between day 1 and 13 of lactation the dry matter content of

the gland was reduced along with mammary fat content while protein content was more than doubled. In the two low protein groups organ size on day 13 lactation tended to be similar or even lower than that on day 1 of lactation, particularly for mammary wet and dry weights. Changes in mammary gland composition were similar to that of the two high protein groups.

Table 2.5. Maternal organ weights and composition on day 1 of lactation of females offered a high (H) or low (L) protein diet from day 12 of gestation.

	DIET OFFERED FROM DAY 12 GESTATION		
	H	L	SD
Liver :			
Wet Weight (g)	16.40	13.02	2.68
Dry Weight (g)	4.12	3.32	0.64
Protein (g)	2.97	2.22*	0.50
Fat (g)	0.67	0.72	0.22
Ash (g)	0.18	0.15	0.17
Mammary Gland :			
Wet Weight (g)	24.99	21.08	4.48
Dry Weight (g)	14.76	12.25	2.66
Protein (g)	2.10	1.81	0.58
Fat (g)	12.09	10.10	2.15
Ash (g)	0.16	0.15	0.03
G.I. Tract :			
Wet Weight (g)	8.78	8.12	0.93
Dry Weight (g)	2.86	2.52	0.59
Protein (g)	0.99	0.95	0.06
Fat (g)	1.67	1.39	0.54
Ash (g)	0.08	0.07	0.00

* $P<0.05$

DISCUSSION

The biphasic nature of protein metabolism during gestation (Naismith *et al.* 1976, 1988) dictates that there is storage of protein in maternal reserves during the first half of pregnancy (anabolic phase) to be utilised in support of the development of the feto-placental unit during the second half (catabolic phase). The changes in carcass protein content of the four treatment groups HH, HL_2, LH and LL_2 represented in Fig. 2.1 support this view of changes in tissue protein masses during gestation.

Table 2.6. Maternal organ weights and composition on day 13 of lactation of the four lactation treatment groups HH, LH, HL_2 and LL_2.

DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION								
	HH (n=6)	LH (n=4)	HL_2 (n=4)	LL_2 (n=5)	SD	DIET EFFECTS GEST.	LACT.	INTER.
Liver :								
Wet Weight (g)	22.67	22.61	14.06	13.47	5.12	-	***	-
Dry Weight (g)	6.49	6.33	3.81	3.61	1.54	-	***	-
Protein (g)	3.85	3.76	2.32	1.94	0.95	-	***	-
Fat (g)	1.70	1.61	0.78	0.95	0.48	-	***	-
Ash (g)	0.23	0.26	0.15	0.13	0.06	-	***	-
Mammary Gland :								
Wet Weight (g)	25.04	28.00	12.38	11.84	7.63	-	***	-
Dry Weight (g)	9.24	8.88	4.31	4.72	2.57	-	***	-
Protein (g)	4.12	4.34	1.83	1.60	1.35	-	***	-
Fat (g)	4.22	3.74	2.17	2.84	1.09	-	***	-
Ash (g)	0.41	0.45	0.16	0.14	0.15	-	***	-
G.I.Tract :						-	***	-
Wet Weight (g)	11.54	12.02	6.90	6.53	2.85	-	***	-
Dry Weight (g)	2.94	3.02	1.60	1.62	0.80	-	***	-
Protein (g)	1.56	1.62	0.88	0.79	0.41	-	***	-
Fat (g)	1.07	1.12	0.53	0.66	0.41	-	***	-
Ash (g)	0.15	0.15	0.09	0.07	0.04	-	***	-

- not significant, *** $P<0.001$

In order to establish differences in the level of maternal protein reserve prior to lactation, the gestational dietary treatment aimed to amplify the catabolism of maternal protein during the second phase by feeding a low protein diet from day 12 onwards. Zartarian *et al.* (1980) had previously reported a significant effect of feeding a 7.5 % protein diet during the second phase on the loss of weight and protein of skeletal muscles in rats.

In the first experiment, the gestational treatments resulted in mean values of maternal protein mass which were different but not significantly so by convention ($P<0.05$). As it was central to the thesis I was exploring that I had confidence the gestational treatments established real differences in maternal protein mass at parturition, data from a second experiment, involving the same gestation treatments, were incorporated into this study. By combining the data for females slaughtered on day 1 of lactation from two parallel experiments it has been shown that the gestational dietary treatments did produce a significant

depletion in maternal carcass protein content prior to lactation ($P<0.01$) (11%). This significant difference in maternal protein reserves on day 1 of lactation might have been greater if the females on the high protein diet could have limited the mobilisation of their carcass protein during the second phase of gestation, but this was not the case even though protein intake was high. It is possible that intakes of the high protein diet during this phase could have been increased if the energy density of the diet had been lower.

However, during lactation group LL_2 were still capable of losing tissue protein (5.8 g). It therefore appears that such dams were able to prevent too great a depletion of maternal reserves during their gestational malnutrition with consequential effects on foetal growth. The significant reduction in foetal birth weight with low protein feeding in gestation supports the view that there is a limit to which foetal parasitism can prevent foetal growth restriction during gestational maternal malnutrition (Anderson *et al.* 1980).

Lactation imposes enormous demands on the body's metabolism and to ensure that lactation proceeds successfully there are co-ordinated adaptations in metabolism (homeorhesis) that partition available nutrients towards the mammary gland and away from tissues which are not essential to lactation (Bauman *et al.* 1980). Along with an elevation of feed intake, physiological changes include hypertrophy of liver, intestines, heart and mammary gland (Williamson 1980). At the same time in well nourished animals there is an expansion of cardiac output and an increase in blood flow to these tissues (Chatwin *et al.* 1969). Thus lactation is associated not only with an increase in mammary size but also other organs involved in supplying nutrients for milk biosynthesis.

In this study the feeding of a low protein diet from day 12 of gestation until parturition generally had no significant affect on organ weight including the mammary gland, although in each case the mean weight was usually less (NS) for L compared with H. This contrasts with a previous study in which dietary protein and energy restriction from day 5 of gestation resulted in a considerable reduction in mammary size by day 21 (Rosso *et al.* 1981).

By day 13 of lactation the feeding of the high protein diet promoted hypertrophy of the major organs associated with lactation, particularly the liver and intestines, in line with an earlier suggestion (Williamson 1980). Although the mammary gland did not show such a marked increase in size it did undergo a distinct change in composition, with its dry weight and fat content declining while its protein content was considerably increased.

The feeding of the low protein diet during lactation prevented any such organ hypertrophy and on day 13 of lactation the liver, mammary and G.I. tract weights of groups HL_2 and LL_2 were significantly lower than the two high protein groups. Blood flow to the mammary gland could also be expected to have been considerably reduced under such dietary conditions (Sakanashi *et al.* 1987). Such a restriction of organ growth in rats has also been reported under conditions of protein/energy restriction (Sakanashi *et al.* 1987) and reductions of protein quality and quantity (Sampson *et al.* 1986). These reductions in organ size associated with the feeding of a low protein diet during lactation reflect the low rates of food consumption achieved by rats offered these diets. Thus the hypertrophy observed in females offered the high protein diet was probably a function of food consumption rather than an inevitable consequence of the state of lactation although, from the pup growth data, intakes of the low protein diet were also associated with impaired lactation.

The comparable feed intakes and lactational performances of the groups offered the high protein lactation diet occurred while group LH were attempting to gain weight and replenish protein reserves. These results show that a high protein diet can be sufficient in allowing litter weight gain not to be hampered by a depletion of tissue reserves which had occurred prior to lactation, and agrees with work by Mahan *et al.* (1975) involving first litter sows.

In earlier studies Sainz *et al.* (1986a) proposed that even females offered a high protein diet will catabolise maternal protein in support of lactation and that this may be influenced by the litter size and dam maturity. The insignificant carcass protein loss of group HH in this study, with a litter size of twelve pups, does not support this proposition. However

it is plausible that in such females tissue reserves are catabolised during the initial stages of lactation before being replenished later on. The measurements of carcass composition made here were insufficiently frequent to check this possibility. It might be suggested, however, that from the evidence presented here there is no obligatory loss of maternal protein during lactation.

The control of tissue protein mobilisation during lactation obviously involves changes in the relative rates of tissue protein degradation and synthesis. From limited work in lactating sheep (Bryant *et al.* 1982, Vincent *et al. 1985*) and rats (Sainz *et al.* 1984) it seems that a rise in degradation is primarily responsible for muscle protein losses during lactation. Subsequent work in this thesis (Chapters 3 and 6) would confirm this.

While in this study the net catabolism of maternal protein reserves during lactation depended on the lactation diet, the loss of body fat deposited during gestation seemed to occur independently of the gestation and lactation diets. While the diets used here were isoenergetic, there were considerable differences in the energy intake during lactation, being 8.34 and 3.52 MJ GE/12d for groups HH and HL_2 respectively, and this was reflected in significant differences in the lactational performance as measured by pup growth. That the rate of net maternal fat loss under these circumstances was similar suggests that fat was being lost from the bodies of these rats at a rate that was close to maximal. A similar loss of fat reserves during lactation is shown by genetically obese rats under conditions of cafeteria feeding (Van Duijuenvoorde *et al.* 1985).

Whether such high rates of fat loss would have been seen if the rats had not been allowed to increase the size of their adipose stores during gestation may be doubtful. Loss of fat during lactation is not obligatory, as thin females can compensate for their lack of fatness by enhancing food intake whilst sustaining equally copious lactation in comparison with fatter comtempories (Garnsworthy 1988). The mass of fat in the body (largely determined by previous nutrition), current nutrition, physiological status and genotype will all play a part in determining both the rate at which fat is lost from the maternal body and the total amount that

can be lost. Whilst the suggestion by Naismith *et al.* (1982) that the catabolism of body stores in under hormonal rather than dietary control is sustained by the results presented here, it is perhaps more appropriate to say that the maximum rate at which these reserves can be lost will be subject to hormonal control. The amount of fat which could be lost at that rate would logically depend on the mass of fat that was present and that will largely be a reflection of previous nutrition. This active partitioning of milk fat precursors would be favoured by the hypoinsulinaemia (Williamson 1980) and high levels of prolactin (Vernon 1989) that are associated with lactation in rodents. Whilst this hypoinsulinaemia and reduced adipocyte responsiveness (Burnol *et al.* 1987) favours the release of fat from the adipose tissue following a shift in the balance of lipogenesis (Williamson 1980) and lipolysis (Smith *et al.* 1976, Zammitt 1988), the enhanced mammary gland insulin responsiveness (Burnol *et al.* 1987) would allow the anabolic processes associated with lactation to be maintained. Reciprocal changes in the adipocyte and mammary gland lipoprotein lipase activity (Hamosh *et al.* 1970, Mendelson *et al.* 1977) further alters the utilisation of circulating lipid and while there is evidence to suggest that prolactin may be involved (Zinder *et al.* 1974), it's action may be indirect and require a functioning mammary gland (Flint *et al.* 1981).

The feeding of a diet inadequate in protein quantity or quality during lactation has been associated with a suppression of feed intake and a reduction in lactational performance in rats (Friggens 1990, Jansen *et al.* 1986, Naismith *et al.* 1982) and also in pigs (Mahan *et al.* 1975), even though the lactating females attempt to support lactation through the catabolism of tissue protein reserves. The results of this current study are in agreement with these previous observations. The situation during lactation presents an interesting contrast to that which can be seen during growth, where animals (for example pigs; Kyriazakis *et al.* 1990) offered highly digestible diets which have a low concentration of protein will attempt to maintain their dietary protein intake by increasing food consumption over that which is seen for similar diets of higher protein content. The consequence of such an action would be, of

course, an increase in the intake of energy yielding nutrients, as well as protein, which would possibly be intolerable in rats that are already mobilising body fat.

During the second half of gestation the feeding of the low protein diet had no significant effect on feed intake. This contrasts markedly with the low intakes achieved in lactation by rats offered the low protein (L_2) diet. An important difference between growing or pregnant rats, and lactating rats, in their response to a food of a low protein:energy ratio is likely to be in the manner in which energy-yielding nutrients are used for fat storage. During both growth and gestation storage of surplus energy-yielding nutrients as fat is both possible and, at least in gestation, even desirable. In lactation where body fat, as here, is being mobilised even when dietary energy intake is high (groups HH and LH), an animal offered a low protein/high energy feed perhaps fails to eat adequately (in terms of protein) because the balance of protein and energy-yielding nutrients which results could create a metabolic embarrassment when associated with the release of fat from the body.

During the first six days of lactation females of group HL_2 had significantly greater intakes and litter weight gains than group LL_2, whilst being able to mobilise significantly more tissue protein. This mobilisation of protein, alongside fat, would have alleviated the imbalance between protein and energy-yielding nutrients which resulted from the combination of diet composition and tissue mobilisation. In rats that were protein depleted at parturition (after receiving diet L during gestation) there was still some tissue protein loss during lactation when diet L_2 was offered. However the extent of this loss was constrained by what appeared to be the lower limit of maternal protein mass. Shields *et al.* (1985) have also reported that during early lactation losses of body protein from first litter sows were significantly reduced by the feeding of a low protein diet (5%) during gestation.

When the gestation diet had had an adequate protein content and maternal protein mass at parturition was relatively high (gestation diet H), subsequent feeding of the low protein diet (L_2) during lactation had a less severe affect on pup growth than when the gestation diet was also low in protein (diet L). Thus it appears that the mobilisation of

maternal protein during lactation was capable of acting as a buffer against dietary protein inadequacy, at least for a while. The stage of lactation at which pup growth of group HL_2 dropped considerably and came to reflect directly diet composition and intake (Fig. 2.3) was possibly the point at which the readily labile tissue reserve approached it's minimum.

The improved lactational performance of group HL_2 during the first six days of lactation was also reflected in a significant alteration in litter composition. The HL_2 litter gained more protein but not fat than LL_2 between day 1 and 13 of lactation. The greater capacity to catabolise labile protein reserves by group HL_2 appeared to allow therefore a significantly improved pup growth both through the extra dietary protein consumed during the first 6 days of lactation (4 g) as well as the use of residual labile protein. This greater lactational performance and improved litter protein gain may not just be the result of alterations in milk yield but also milk composition (or both), although no measurements of milk composition were made.

The carcass protein contents on day 13 lactation (Fig. 2.1) were possibly reached before this point and these females could be approaching the limit of their protein reserves. From the patterns of litter growth (Fig. 2.3) it might be reasonable to suggest that the support of litter growth by the mobilisation of maternal protein reserves was exhausted by day 6 or 7 of lactation in dams of group HL_2. If day 6/7 was the point at which the bulk of tissue labile protein reserves are expended and thus its impact on lactation was curtailed, the balance of tissue protein metabolism would need to be adjusted to prevent further mobilisation of tissue protein. The controlling factors involved in such a mechanism remain to elucidated. It is of interest to note the similar carcass protein content of the HL_2 and LL_2 females on day 13 lactation, which could represent the limit to which tissue reserves could be catabolised (Glore *et al.* 1985). In these animals this was approximately 72 % of the carcass protein on day 2 of gestation and suggests that during the whole period of reproduction these females lost approximately 28 % of carcass protein, close to the 25 % suggested by Allison *et al.* (1965).

The results of the current study confirm the findings of Naismith and co-workers that although lean tissue can be catabolised during lactation in response to dietary protein restriction, the supply of endogenous protein is insufficient to allow lactation to continue at the level of similar females receiving an adequate protein supply, at least not beyond the first few days of lactation. It may also be concluded that the mobilisation of endogenous adipose stores tends to suppress the intake of a low protein diet. However I have extended the findings of Naismith and co-workers by presenting evidence that suggests assumptions concerning the extent of protein reserve repletion at parturition can under or over estimate a females ability to respond to inadequate dietary protein during lactation. These results also confirm that females can actively regulate the loss of protein from carcass reserves during gestation and lactation.

In summary it may be concluded that the utilisation of maternal protein reserves during lactation can improve lactational performance under conditions of dietary protein inadequacy when intake is suppressed by the loss of maternal adipose stores. However this influence is constrained by the extent of the maternal reserves available and the capacity of females to mobilise such reserves. The depletion of protein reserves prior to lactation does not inhibit lactational performance when an adequate supply of dietary protein is provided. In fact a more efficient use of the dietary protein could occur as females attempt to replenish depleted reserves while maintaining lactational performance.

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CHAPTER THREE

EXPERIMENT E2

EFFECTS of DIETARY PROTEIN RESTRICTION DURING GESTATION and LACTATION on TISSUE PROTEIN METABOLISM and Na^+,K^+-ATPase ACTIVITY in LACTATING RATS.

Effects of Dietary Protein Restriction During Gestation and Lactation on Tissue Protein Metabolism and Na^+,K^+-ATPase Activity in Lactating Rats

ABSTRACT

Changes in tissue protein synthesis and an associated membrane transport system in rats were investigated during lactation and under conditions of dietary protein restriction. Following mating, multiparous female Sprague-Dawley rats were caged individually and offered a high protein diet (H; 215 g CP/kg DM) *ad libitum* until day 12 gestation. Subsequently half continued to receive diet H, whilst the remainder were offered a low protein diet (L; 65 gCP/kg DM) until parturition. On day 1 of lactation females were then allocated to either diet H or another low protein diet (L_2; 90 gCP/kg DM) which were offered *ad libitum* until day 13 of lactation, giving four lactation groups HH, LH, HL_2 and LL_2. On day 1 and 13 of lactation groups of females were used in the estimation of tissue protein synthesis (flooding dose of [^{3}H] phenylalanine) and Na^+,K^+-ATPase activity (polarographically) in skeletal muscle, mammary gland, liver and duodenal mucosa. By day 1 of lactation, diet L had reduced rates of muscle protein synthesis ($P<0.05$) and the O_2 consumption associated with Na^+,K^+-ATPase, although not significantly ($P<0.10$). Rates of protein synthesis in the other tissues studied were not affected on day 1 of lactation by the gestation dietary treatment. By day 13 of lactation, the feeding of diet L_2 had reduced the muscle FSR and ASR of group HL_2 to rates that were lower than that on day 1 ($P<0.05$), comparable to that of group LL_2 and lower than that of groups HH and LH ($P<0.05$). Diet H had allowed group LH to increase their muscle protein synthesis compared to that on day 1 ($P<0.05$). The muscle Na^+,K^+-ATPase activity on day 13 of lactation was also lower in groups offered diet L_2 ($P<0.05$). Mammary protein synthesis was increased during lactation with the feeding of diet H ($P<0.05$), which was prevented by diet L_2 such that rates of groups HL_2 and LL_2 were lower than that of the two high protein groups on day 13 ($P<0.01$). Mammary

respiration and in particular Na^+,K^+-ATPase activity was increased during lactation by the feeding of diet H ($P<0.05$). Rates of protein synthesis and respiration in the liver and duodenal mucosa were not significantly affected by the gestational or lactational dietary treatments. Calculated rates of muscle protein degradation suggest that whilst the loss of muscle protein in group HL_2 during lactation might have been promoted by the decline in synthesis, the increase in degradation may be quantitatively more important.

INTRODUCTION

During lactation the associated increase in a female's nutrient requirements are usually met through an elevation of feed intake. Restrictions in the supply of dietary protein and energy can significantly impair milk production in humans (Sampson *et al.* 1984a), rats (Naismith *et al.* 1982) and pigs (Mullan *et al.* 1989a), and under such conditions lactating females attempt to support milk production by mobilising body reserves of protein, the use of which has been reported for cattle (Botts *et al.* 1979), sheep (Lynch *et al.* 1988) humans (Motil *et al.* 1989) and rats (Naismith *et al.* 1987, Chapter 2). The degree to which such reserves can support milk production is however limited and also influenced by the extent of their repletion at parturition (Chapter 2).

A female's labile protein reserves are found predominantly in the skeletal muscle (Swick *et al.* 1977) and clearly protein mobilisation occurs when the balance of protein turnover is such that the rate of degradation exceeds that of synthesis. However, unlike the clearly established changes in adipocyte metabolism associated with the mobilisation of lipid stores during lactation (Bauman *et al.* 1980, Vernon 1989, Williamson 1980), changes in tissue protein metabolism involved in partitioning available amino acids towards the mammary gland remain uncertain and confused.

Changes in muscle protein turnover of non-lactating animals in response to a reduction in protein supply have received considerable attention and are now well established, involving a rapid fall in synthesis followed later by an increase in degradation (Millward *et al.*

1978). However in comparison, information concerning lactating animals is limited. For lactating ruminants, some workers have reported that in goats the loss of muscle mass and protein associated with early lactation is primarily the result of a reduction in protein synthesis (Baracos *et al.* 1991), while others working with lactating sheep have implicated an elevation in muscle protein degradation as the major mechanism involved (Vincent *et al.* 1985). Bryant and Smith (1982) on the other hand have suggested that in lactating sheep the mobilisation of muscle protein resulted from a decrease in synthesis or an increase in degradation which depended upon the individual muscle considered. In lactating rodents subjected to conditions that promoted carcass protein loss, no alterations in whole body protein synthesis or degradation could be detected (Sainz *et al.* 1986b). However, in that instance measurement of whole body rates of protein metabolism may have masked any changes that occurred in individual tissues. In lactating females offered an adequate dietary protein supply that prevented any great depletion of maternal protein, rates of muscle protein synthesis were similar to that in non lactating females (Millican *et al.* 1987, Siebrits *et al.* 1985). Therefore conclusions on changes in muscle protein turnover involved in protein loss are varied and the subject of some disagreement.

Whole body protein turnover is considerably increased during lactation, primarily due to an increase in protein synthesis (both fractional and absolute) of the mammary gland, liver and G.I. tract. Mammary protein synthesis increases from early to peak lactation (Jansen *et al.* 1986, Millican *et al.* 1987) and has been shown to be highly correlated with milk secretion (Sampson *et al.* 1985), although the protein synthesised within the gland includes structural, enzymatic and milk proteins. Protein synthesis in the mammary gland is significantly influenced by changes in dietary protein quantity and quality (Sampson *et al.* 1986), while synthesis in the liver is thought to be less sensitive during lactation to such variations in protein supply (Jansen *et al.* 1986).

Various cellular processes are involved in supporting protein synthesis. One such process is the membrane transport mechanism Na^+,K^+-ATPase (EC 3.6.1.3) which has been

reported to be closely associated with muscle protein synthesis (Adeola *et al.* 1989, Vandenburgh *et al.* 1981). The Na^+,K^+-ATPase enzyme is primarily involved in the maintenance of cellular ionic homeostasis (Na^+, K^+) along with the transport of amino acids and sugars across cell membranes. The support of the Na^+,K^+-ATPase activity is a major component of cellular energy expenditure, being 20% or more of total O_2 uptake by tissues (Milligan *et al.* 1985). This activity is altered by physiological status and has been shown to be significantly increased in the duodenal mucosa and liver of ruminants during lactation (McBride *et al.* 1984, 1985a).

It has been previously reported in this thesis that the extent of maternal protein repletion prior to lactation significantly influences a female's ability to sustain milk production when dietary protein supply is limiting, while previous maternal protein depletion had no effect on lactational performance as long as an adequate supply of dietary protein was provided (Chapter 2). Such females also attempted to replenish their depleted protein reserves with the additional dietary protein offered during lactation.

The objective of the current study was to investigate the changes in muscle protein metabolism involved in protein mobilisation and possible replenishment during lactation, along with changes in associated membrane transport processes.

MATERIALS and METHODS

The experiment reported here adopted a similar experimental protocol to that reported in Experiment E1 (Chapter 2), in which the effect on lactational performance of variations in the extent of maternal protein reserve repletion was investigated. The objective of the experimental protocol was to establish at parturition two groups of females that had distinct differences in the size of their maternal protein mass and thus reserves. The experimental protocol is described in detail below.

Experimental Design

Forty four multiparous female Sprague-Dawley rats (Harlan and Olac UK Ltd.) weighing on average 302.3 (± 2.1)g were caged individually in a room regulated at 22 °C and relative humidity from 40 - 60 %, under a 12 hour light-dark cycle with the light period from 0800 - 2000 hours. For mating the females were placed, at the appropriate time, individually in a wire bottomed cage with a proven male breeder. The morning on which mating was confirmed, through the presence of vaginal plugs, was designated day 1 of gestation and the females were returned to solid bottomed plastic cages for the remainder of the experiment.

Following mating the females were offered a high protein diet (H, 215 gCP/kg DM) (Table 3.1) *ad libitum* until day 12 of gestation. Subsequently half of the females continued to receive the high protein diet while the remainder were offered a low protein diet (L, 65 gCP/kg DM) *ad libitum* until parturition. Immediately following parturition four groups of females were selected for slaughter, two groups to be used in carcass analysis (n=4) while the other two groups (n=6) were used in the analysis of tissue metabolism (protein synthesis and Na^+,K^+-ATPase activity) (see below).

Table 3.1. Diet formulation (g/kg DM).

	HIGH (H)	LOW (L)	LOW (L_2)
Casein[1]	215	65	90
Starch/Sucrose[2]	439	542	525
Vegetable Fat	196	243	235
Vitamin Mix[3]	50	50	50
Mineral Mix[3]	100	100	100

[1] Casein supplemented with DL-Methionine (99% + 1%)
[2] Starch and Sucrose mixture in ratio 2 : 1
[3] Vitamin and Mineral mix formulated to meet N.R.C. requirements 1978
Anti-oxidant (Butylated hydroxy toluene): 0.001% Fresh Matter
Diet Analysis : Protein (g CP/kg DM); H 212, L 63, L_2 88
GE (MJ/kg DM); H 21.70, L 21.54, L_2 21.69

On day 1 lactation the remaining females were allocated factorially to either diet H or a low protein diet (L_2, 90 gCP/kg DM) which were then offered *ad libitum* for the remainder of the experiment. This dietary allocation produced four groups of females (HH,

HL_2, LH and LL_2, the first letter representing the dietary treatment from day 12 gestation and the second letter representing the lactation diet) that reached day 13 of lactation, on which all females and litters were slaughtered and measurements of maternal tissue metabolism were made (see below).

All diets were formulated to provide approximately 21 MJ GE/kg DM with a constant carbohydrate:fat ratio of 2.4:1. All litters were standardised to 12 pups on day 1 of lactation to maximise the lactational stress imposed, and litter weights were recorded daily throughout lactation. Dam body weights and feed intakes were recorded throughout the experiment. All females were given free access to drinking water.

Measurement of Protein Synthesis

On either day 1 or 13 of lactation rates of total protein synthesis were measured *in vivo* in the mammary gland, liver, gastrocnemius muscle and duodenal mucosa using the flooding dose technique of Garlick *et al.* (1980) (Appendix 1).

Between 0900 and 1300 hours dams were injected via a lateral tail vein with a solution containing 150 mM L-phenylalanine and 50 μCi/ml L-[2, 6 ^{3}H] phenylalanine (Amersham) at 1.0 ml/100 g body weight and returned to their litters. After 10 minutes dams were decapitated and samples of the left inquinal abdominal mammary gland, liver, gastrocnemius muscle and duodenal mucosa were quickly excised and plunged into liquid nitrogen. A sample of mucosa was obtained following opening of a length of duodenum, washing with ice cold saline and scraping with a microscope slide. In this study the gastrocnemius muscle was used because it is thought to be a good indicator of the response of the body's musculature to dietary treatment (Waterlow *et al.* 1978). Samples of tissue were used to measure Fractional Synthesis Rates (FSR) of total tissue protein from the incorporation of [^{3}H] phenylalanine into tissue protein. Correction for the gradual linear decline of specific activity of tissue free phenylalanine during the 10 minute incorporation period was ignored because of the previous observation that the rate of decline in mammary

tissue, liver and muscle is slow and insignificant (Garlick *et al.* 1983, Sampson *et al.* 1986). Calculation of FSR (percent/day) uses the formula:

$$FSR = \frac{S_B \times 100}{S_A \times t}$$

where S_B and S_A are specific activities of protein bound and free phenylalanine respectively and t is the time in days that elapsed between injection and rapid cooling of tissue. Absolute synthesis rates (ASR) are calculated from FSR and tissue protein content. Tissue RNA concentration was measured as described by Munro *et al.* (1969), with muscle RNA calculated using the equation of Ashford *et al.* (1986), and tissue protein concentration was measured with the method of Lowry *et al.* (1951) using bovine serum albumin as a standard.

Measurement of Na^+,K^+-ATPase Activity

The tissue activity of the enzyme Na^+,K^+-ATPase on day 1 and 13 of lactation was estimated through measurements of the inhibition of tissue oxygen consumption caused by the addition of ouabain, a specific inhibitor of the Na^+,K^+-ATPase enzyme (Albers *et al.* 1968), to the incubation medium. Ouabain concentrations of 1 μM or greater have been shown to give maximal inhibition of Na^+,K^+-ATPase associated respiration (Gregg *et al.* 1982a).

Oxygen consumption rates were measured polarographically using a Rank oxygen electrode (Appendix 1). Weighed samples of liver snips, mammary gland slices (20 μm) (Bartley *et al.* 1976), muscle fibre bundles (tied with sutures) (Gregg *et al.* 1982b) and duodenal mucosal scrapes were washed and placed in a rank oxygen electrode chamber containing 3 ml of Minimal Essential Medium (MEM) containing 5mM HEPES at 37 °C pH 7.4, and the oxygen consumption was recorded for 10 - 15 min. After this time ouabain was added to a final concentration of 10^{-4} M and the oxygen consumption was recorded for a further 10 min. The difference between the initial oxygen consumption and that following ouabain treatment was termed the Na^+,K^+-ATPase dependent respiration. The percentage

inhibition of the original oxygen consumption associated with Na^+,K^+-ATPase activity was calculated using the ratio of this to the initial oxygen consumption.

Carcass Analysis

For the groups selected for carcass analysis on day 1 of lactation, dams were killed by decapitation and their carcass, liver, mammary gland and gastrointestinal tract were dissected and analysed for dry matter, protein, ash and fat. The procedures used for these analyses have been described elsewhere (Chapter 2, Appendix 1).

Statistical Analysis

For the statistical treatment of the results two- way, one-way analysis of variance and by calculation of least significant differences, T-Test were used to compare sample means between diets and stages of lactation. To establish the impact that the gestation treatments had on the extent of maternal protein reserve repletion at parturition, the carcass composition data for dams slaughtered on day 1 of lactation were amalgamated with those for rats from a parallel experiment (E1, Chapter 2) and treated similarly during lactation.

RESULTS

Feed Intakes and Body Weight Changes During Gestation

The feed intakes, body weight and carcass composition changes, mean pup birth weights and pups/litter of females offered diets H or L during gestation are shown in Table 3.2. The feeding of the low protein diet during the second half of gestation did not significantly affect the feed intake or maternal gestation weight gain compared to females offered the high diet. The low protein dietary treatment also did not impair foetal development, and the mean pup birth weight and litter size (pups/litter) did not differ between the two treatment groups.

Table 3.2. Effect of gestation dietary treatment on maternal body weight gain, carcass composition, gestational feed intakes and pup birth weight.

DIETARY TREATMENT	H (n=21)	L (n=21)	SD
Dam Weight Gain (g)	12.0	3.3	19.5^{NS}
Feed Intake (g DM) day 1-11	193.8	193.8	16.7^{NS}
Feed Intake (g DM) day 11-Part.	182.3	186.8	24.8^{NS}
Carcass Composition[1] D1 Lact. :			
Protein	43.5	38.7	3.6^{**}
Fat	22.8	23.5	5.8^{NS}
Abdominal Fat	19.6	21.1	6.1^{NS}
Mammary Weight[2] (g)	15.0	14.3	4.2^{NS}
Protein (g)	1.6	1.4	0.3^{NS}
Fat (g)	5.5	6.2	2.1^{NS}
Liver Weight[2] (g)	11.2	10.7	1.4^{NS}
Protein (g)	2.2	1.9	0.2^{NS}
Fat (g)	0.9	1.1	0.3^{NS}
G.I. Tract Weight[2] (g)	7.9	8.2	1.6^{NS}
Protein (g)	1.1	1.2	0.2^{NS}
Fat (g)	1.0	1.3	0.7^{NS}
Litter Size (pups/litter)	12.8	11.9	3.2^{NS}
Mean Pup Birth Weight (g)	5.7	5.4	0.8^{NS}

1 Composition Estimated using data from parallel Experiment (H, n=8; L, n=7)
2 (H, n=4; L, n=4)
NS Non Significant, * $P<0.05$, ** $P<0.01$.

It has already been indicated that in order to provide a stronger indication of the extent of maternal protein reserve repletion at parturition, the effects of the gestation dietary treatments on the carcass composition of females slaughtered on day 1 of lactation were estimated using data from two parallel experiments. From Table 3.2 it can seen that the feeding of the low protein diet during the second half of gestation had reduced ($P<0.01$) the carcass protein content and thus protein reserves of group L, while having no significant effect on the size of their carcass and abdominal fat stores. The gestation dietary treatment had therefore the desired effect of ensuring that females at parturition had variations in the size of their protein reserve.

Dietary protein restriction during gestation also had no significant effect on the size and composition of the mammary gland, liver and G.I. tract on day 1 of lactation.

Effects of the Gestational and Lactational Dietary Treatments on Dam Feed Intakes, Body Weight Changes and Litter Weight Gains During Lactation

The results for the four lactation groups HH, LH, HL_2 and LL_2 are shown in Table 3.3. The changes in maternal body weight during lactation were significantly affected only by the lactational dietary treatment. All four groups lost weight, but the feeding of the low protein diet resulted in a greater weight loss ($P<0.05$), although the weight loss by groups HL_2 and LL_2 were not significantly different and this reflects their similar weight gains during gestation. The weight loss of the two high protein groups were also not significantly different.

Table 3.3. Effect of gestation and lactation dietary treatments on maternal body weight losses, feed intakes and litter weight gains during lactation.

DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION								
	HH (n=6)	LH (n=5)	HL_2 (n=6)	LL_2 (n=6)	SD	DIET EFFECTS GEST.	LACT.	INTER.
Dam Weight Change (g/12d)	-17.4	-6.6	-63.1	-51.6	28.0	-	**	-
Feed Intake (g/DM/12d)	337.2	292.9	256.8	226.7	53.5	*	***	-
Day 1-6 (g DM)	130.5	103.0	126.5	104.8	19.5	**	-	-
Day 7-13 (g DM)	206.7	189.9	130.3	121.9	45.6	-	***	-
Litter Weight Gain (g/12d)	193.7	153.5	94.0	69.1	52.5	***	***	-
Day 1-6	81.8	52.4	52.2	31.7	21.6	***	***	-
Day 7-13	111.9	101.1	41.8	37.4	35.2	*	***	-

DM Dry Matter

- not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

The feed intakes of the four treatment groups were influenced by both gestational and lactational dietary treatments. The total feed intake (g DM/12 d) of diet H by group LH was less than that of group HH ($P<0.05$). This difference in total feed intake between the two groups offered diet H was due to the lower intake of group LH during the first six days ($P<0.05$). Both groups HH and LH showed a considerable increase in feed intake between the two halves of lactation. The feeding of diet L_2 during lactation resulted in a suppression ($P<0.05$) in total feed intake of groups HL_2 and LL_2 when compared to group HH. This reduction in feed intake was greatest for group LL_2; intake by group LL_2 being lower ($P<0.05$) than that for group HL_2 during the first six days and whole 12 day period. During the first half of lactation intake of diet L_2 by group HL_2 was greater than intake of group LH ($P<0.05$) but not that of the group HH. During the second half of lactation intakes of both high groups had increased sufficiently to be greater than that of HL_2 ($P<0.05$).

Lactational performance was estimated by the weight gain of a standardised litter and was influenced by both gestation and lactation dietary treatments. The lactational performance of group LH was less ($P<0.05$) than that of group HH, reflecting the difference in their intakes of diet H. For groups HH and LH, litter weight gain increased between the two halves of lactation, again reflecting the considerable increase in feed intake. Dietary protein restriction significantly impaired the litter weight gain of groups HL_2 and LL_2 when compared to HH and LH. However the lactational performance of the low protein groups was influenced by their gestation dietary treatment, with group LL_2 having a lower litter weight gain (total and first six days) than group HL_2 ($P<0.01$). Group HL_2's greater performance during the first half of lactation was supported by their greater feed intake and maternal protein reserves, and allowed them to achieve a similar lactational performance to that of group LH. Despite this, group HL_2 were unable to maintain this greater performance and their rate of litter weight gain was reduced by 20% during the second half of lactation.

Effect of Gestational Dietary Treatment on Tissue Protein Synthesis on Day 1 of Lactation

Rates of protein synthesis and tissue composition for the gastrocnemius muscle, mammary gland, liver and duodenal mucosa for females offered diet H or L during the second half of gestation are shown in Table 3.4.

Table 3.4. Effect of gestation dietary treatment on rates of tissue protein synthesis on day 1 of lactation.

DIETARY TREATMENT	H	L	SD
Muscle :			
Wet Weight (g)	1.70	1.40	0.2^{***}
Protein (mg)	345.33	290.79	48.0^{**}
RNA (mg)	2.21	1.75	0.3^{***}
FSR (%/d)	4.4	3.4	0.8^{*}
ASR (mg protein/d)	15.0	9.9	3.4^{*}
RNA Activity (mg Prot/mg RNA)	6.84	5.70	1.4^{NS}
Mammary Gland :			
FSR (%/d)	58.9	60.8	10.9^{NS}
ASR (mg/d/g Tissue)	52.4	47.4	14.5^{NS}
Protein (mg/g Tissue)	89.00	76.77	13.8^{NS}
RNA (mg/g Tissue)	4.66	3.75	0.9^{NS}
RNA Activity (mg Prot/mg RNA)	11.89	12.50	3.6^{NS}
Liver :			
FSR (%/d)	96.5	103.0	18.1^{NS}
ASR (mg/d/g Tissue)	190.2	190.0	24.5^{NS}
Protein (mg/g Tissue)	197.59	188.62	20.9^{NS}
RNA (mg/g Tissue)	12.74	11.45	1.0^{NS}
RNA Activity (mg Prot/mg RNA)	15.38	16.42	2.0^{NS}
Duodenal Mucosa :			
FSR (%/d)	115.7	131.8	22.0^{NS}
ASR (mg/d/g Tissue)	118.7	128.1	25.9^{NS}
Protein (mg/g Tissue)	102.2	97.33	12.9^{NS}
RNA (mg/g Tissue)	6.71	5.73	1.4^{NS}
RNA Activity (mg Prot/mg RNA)	17.99	22.98	3.9^{NS}

FSR Fractional Synthesis Rate (%/d)
ASR Absolute Synthesis Rate (mg Protein/d)
NS Non Significant, * P<0.05, ** P<0.01, *** P<0.001.

The feeding of diet L resulted in a reduction in muscle weight (P<0.001), protein content (P<0.01) and RNA content (P<0.001) by day 1 of lactation. The rates of muscle protein synthesis were also reduced (P<0.05) by the low protein dietary treatment, with

muscle FSR (%/day) and ASR (mg Protein/day) being reduced from 4.4 %/d and 15.0 mg/d to 3.4 %/d and 9.9 mg/d in groups H and L respectively. There was no significant difference in muscle RNA activity between groups H or L. The reduction in gastrocnemius muscle weight and protein content, by 18 and 16 % respectively, of group L females reflects the loss of maternal protein reserves, which is also indicated by the reduced carcass protein content of such females (Table 3.2).

The rate of protein synthesis and tissue composition in the mammary gland, liver and duodenal mucosa on day 1 of lactation was not significantly affected by gestation dietary treatment (Table 3.4), and reflects the lack of effect of such dietary treatment on organ size (Table 3.2). The main effect of the low protein dietary treatment on the depletion of maternal protein reserves was therefore reflected in muscle protein metabolism.

Effect of Gestational Dietary Treatment on Tissue O_2 Consumption and Na^+,K^+-ATPase-Dependent Respiration

The oxygen consumption and associated Na^+,K^+-ATPase activity of the muscle, mammary gland, liver and duodenal mucosa on day 1 of lactation are shown in Table 3.5.

Dietary protein restriction during the second half of gestation tended to reduce the Total, Na^+,K^+-ATPase-Dependent and Independent respiration of the tissues studied by day 1 of lactation, although with the exception of the liver Total O_2 consumption ($P<0.05$) these reductions were not statistically significant at the 5% level.

In the gastrocnemius muscle the proportion of O_2 consumption inhibited by ouabain (Na^+,K^+-ATPase-Dependent respiration) tended to be lower ($P<0.10$) for group L than for group H and ranged from 24 - 32.3 %. This proportion in the mammary gland, liver and duodenal mucosa ranged from 12.5 - 15.7 %, 18.9 - 21.1 % and 17.6 - 20.3 % respectively.

Table 3.5. Effect of gestation dietary treatment on tissue respiration (nmoles O_2/g tissue/min) and the proportion associated with Na^+,K^+-ATPase activity (ouabain sensitive respiration) on day 1 of lactation.

DIETARY TREATMENT	H	L	SD
Muscle :			
Total Respiration	312.3	254.2	108.8^{NS}
Ouabain Insensitive	218.7	196.8	102.4^{NS}
Ouabain Sensitive	93.6	57.4	32.9^{*}
% Inhibition	32.3	24.0	10.2^{*}
Mammary Gland :			
Total Respiration	517.6	346.4	179.9^{NS}
Ouabain Insensitive	445.4	303.2	147.4^{NS}
Ouabain Sensitive	72.2	43.2	24.7^{NS}
% Inhibition	15.7	12.5	7.4^{NS}
Liver :			
Total Respiration	1414.8	1063.0	308.2^{**}
Ouabain Insensitive	1099.1	860.8	191.6^{*}
Ouabain Sensitive	315.7	202.2	145.4^{NS}
% Inhibition	21.1	18.9	6.5^{NS}
Duodenal Mucosa[1] :			
Total Respiration	3.6	3.1	1.6^{NS}
Ouabain Insensitive	3.0	2.5	1.4^{NS}
Ouabain Sensitive	0.6	0.6	0.3^{NS}
% Inhibition	17.6	20.3	6.9^{NS}

[1] Mucosal Respiration nmoles O_2/mg Protein/min.
NS Not Significant, * $P<0.10$, ** $P<0.05$.

The Effects of Gestational and Lactational Dietary Treatments on Tissue Protein Synthesis and O_2 Consumption on Day 13 Lactation

Muscle: The rate of muscle protein synthesis, muscle composition and tissue respiration of the four lactation treatment groups HH, LH, HL_2 and LL_2 on day 13 of lactation are shown in Table 3.6.

The rate of muscle protein synthesis on day 13 of lactation was influenced solely by the lactational dietary treatment, with both FSR and ASR of groups HL_2 and LL_2 being lower ($P<0.05$) than that of the two high protein groups. The reduced rates of muscle protein synthesis, both FSR and ASR, of group HL_2 were also lower ($P<0.05$) than that of group H on day 1, being reduced from 4.40 to 3.34 %/d and 15.05 to 8.44 mg/d respectively. A

similar effect is seen for group LH, where feeding of diet H increased ($P<0.05$) muscle protein synthesis when compared to that of group L on day 1, increasing from 3.40 to 4.84 %/d and 9.87 to 14.55 mg/d for FSR and ASR respectively. The rate of muscle protein synthesis in groups HH and LL_2 did not appear to change through lactation.

Table 3.6. Effect of gestational and lactational dietary treatments on muscle protein synthesis, muscle composition and O_2 consumption (nmoles O_2/g tissue/min) on day 13 of lactation.

DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION								
						DIET EFFECT		
	HH	LH	HL_2	LL_2	SD	GEST.	LACT.	INTER.
Wet Weight (g)	1.60	1.43	1.26	1.28	0.2	*	***	**
Protein (mg)	330.58	301.22	252.00	245.45	40.8	-	***	-
RNA (mg)	1.99	1.89	1.41	1.54	0.3	-	***	-
FSR (%/d)	4.9	4.8	3.3	3.9	0.8	-	**	-
ASR (mg Prot/d)	16.0	14.5	8.4	9.7	3.6	-	***	-
RNA Activity	8.13	7.83	5.99	6.30	1.5	-	**	-
O_2 Consumption								
Total Respiration	264.2	272.1	234.2	243.9	52.4	-	-	-
Ouabain Insensitive	164.8	178.1	166.3	173.9	32.4	-	-	-
Ouabain Sensitive	99.4	94.0	67.9	70.0	30.2	-	*	-
% Inhibition	36.7	33.6	29.3	28.4	6.4	-	*	-

FSR Fractional Synthesis Rate
ASR Absolute Synthesis Rate
RNA Activity mg protein/mg RNA
- not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Dietary protein restriction during lactation reduced ($P<0.05$) muscle weight, protein and RNA contents of groups HL_2 and LL_2 when compared to that of groups HH and LH. These reduced muscle protein contents were also lower ($P<0.05$) than that of groups H and L on day 1 of lactation, with muscle protein losses of approximately 93 and 45 mg between day 1 and 13 for HL_2 and LL_2 respectively. Muscle weight and RNA content of group HL_2 were also lower ($P<0.05$) than that of group H on day 1 of lactation. The muscle weight, and protein content of group LH were lower ($P<0.05$) than that of group HH on day 13 lactation and were not significantly greater than values for group L on day 1. Group LH were thus unable to replenish their depleted muscle protein when offered the high protein diet despite the fact that their protein synthetic rate and RNA activity were increased compared to day 1 of

lactation. Group HH showed no change in their muscle protein metabolism or composition compared to group H on day 1.

On day 13 of lactation, muscle energy expenditure, represented by Total O_2 consumption, showed no significant effect of gestation or lactation dietary treatments, although the O_2 consumption by the groups offered diet L_2 tended to be lower than that of the two high protein groups (Table 3.6). This lower muscle O_2 consumption by HL_2 and LL_2 was reflected in a significantly reduced Na^+,K^+-ATPase-Dependent respiration compared to that of the groups offered diet H, with this difference being significant at the 10% level between group HH and the two low groups. When these results are presented as the proportion of Total O_2 consumption (% Inhibition), the proportion associated with Na^+,K^+-ATPase activity was lower ($P<0.05$) in groups HL_2 and LL_2 when compared to that of group HH. On day 13 of lactation Na^+,K^+-ATPase activity accounted for between 28 - 37% of muscle respiration. There were no significant changes in total muscle respiration between day 1 and 13 of lactation although it can be seen that dietary protein restriction reduced the proportion associated with Na^+,K^+-ATPase (Table 3.6).

Mammary Tissue: The rates of mammary protein synthesis, mammary composition and tissue respiration for the four treatment groups HH, LH, HL_2 and LL_2 on day 13 of lactation are shown in Table 3.7.

The feeding of diet H during lactation promoted an increase ($P<0.05$) in mammary protein synthesis when compared to that on day 1 of lactation, with FSR increasing from around 59 %/d on day 1 to 92 and 82 %/d on day 13 for groups HH and LH respectively. Dietary protein restriction during lactation prevented any substantial increase in mammary protein synthesis of groups HL_2 and LL_2, except for the ASR of HL_2 ($P<0.05$), and resulted in the two low protein groups having lower ($P<0.01$) rates of mammary protein synthesis, both FSR and ASR (mg protein/g tissue/day), when compared to that of groups HH and LH.

Table 3.7. Effect of gestation and lactation dietary treatment on mammary gland protein synthesis, composition and O_2 uptake (nmoles O_2/g tissue/min) on day 13 of lactation.

DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION								
						DIET EFFECT		
	HH	LH	HL_2	LL_2	SD	GEST.	LACT.	INTER.
FSR (%/d)	91.6	82.4	58.9	59.3	20.0	-	**	-
ASR (mg/g/d)	118.2	113.5	74.1	64.7	28.6	-	**	-
Protein (mg/g)	130.08	139.30	124.30	109.92	16.2	-	**	-
RNA (mg/g)	11.40	10.37	8.62	7.92	2.0	-	**	-
RNA Activity	10.68	10.94	8.58	8.09	2.0	-	**	-
O_2 Consumption:								
Total Respiration	594.0	642.2	515.8	361.4	169.3	-	**	-
Ouabain Insensitive	467.0	499.9	408.3	286.2	147.4	-	**	-
Ouabain Sensitive	127.0	142.3	107.5	75.2	44.7	-	*	-
% Inhibition	22.3	22.8	20.0	21.9	7.4	-	-	-

FSR Fractional Synthesis Rate
ASR Absolute Synthesis Rate (mg/ g Tissue/d)
RNA Activity mg Protein/mg RNA
- not significant, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

Lactation resulted in a change in mammary composition, with the protein and RNA contents (mg/g Tissue) increasing ($P<0.05$) in all groups when compared to day 1, although protein restriction of group LL_2 prevented as great an increase as that of groups HH and LH (Table 3.7). On day 13 of lactation the protein content, RNA content and activity of the groups offered diet L_2 were significantly lower than those offered the high protein diet during lactation. The RNA activity of the four treatment groups tended to be lower than that of groups H and L on day 1, with the reduction in RNA activity of the LL_2 group being significant ($P<0.05$) (Table 3.4).

On day 13 of lactation the mammary Total, Na^+,K^+-ATPase-Dependent and Independent respirations were not significantly different for groups HH, LH and HL_2, although diet L_2 resulted in group LL_2 having lower ($P<0.05$) levels of respiration compared to the two high protein groups. Both low protein groups showed no significant increase in mammary respiration during lactation. However the feeding of the high protein diet during lactation resulted in an increased mammary O_2 consumption (Table 3.7 & Table 3.5). The increase in Total and Na^+,K^+-ATPase-Independent respiration was significant for only group LH ($P<0.05$), while the Na^+,K^+-ATPase-Dependent respiration was significantly increased in

both HH and LH groups ($P<0.05$). By day 13 of lactation, the proportion of total respiration associated with Na^+,K^+-ATPase activity was increased ($P<0.05$) in both LH and LL_2 groups compared to that on day 1, accounting for between 20 - 23% of total mammary respiration (Table 3.7).

Liver: On day 13 of lactation, the gestational and lactational dietary treatments had no significant effect on hepatic protein synthesis, with both liver FSR and ASR (mg protein/g Tissue/day) not different for the four lactation treatment groups (Table 3.8). These rates were also not significantly different from those on day 1 of lactation (Table 3.4).

Table 3.8. Effect of gestation and lactation dietary treatments on liver protein synthesis, composition and O_2 Consumption (nmoles O_2/g tissue/min) on day 13 of lactation.

DIETARY PROTEIN SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION.								
						DIET EFFECT		
	HH	LH	HL_2	LL_2	SD	GEST.	LACT.	INTER.
FSR (%/d)	92.9	85.5	98.2	103.5	16.5	-	-	-
ASR (mg/g/d)	183.1	165.3	161.6	159.3	30.8	-	-	-
Protein (mg/g)	196.92	193.80	164.25	151.39	21.7	-	**	-
RNA (mg/g)	8.37	8.51	8.66	7.59	0.8	-	-	-
RNA Activity	21.96	19.40	18.50	20.96	3.2	-	-	-
O_2 Consumption:								
Total Respiration	1087.5	1007.5	872.3	891.4	218.1	-	-	-
Ouabain Insensitive	866.2	820.6	696.1	739.4	178.8	-	-	-
Ouabain Sensitive	221.3	186.9	176.2	152.0	68.3	-	-	-
% Inhibition	20.6	18.4	20.0	16.9	4.9	-	-	-

FSR Fractional Synthesis Rate
ASR Absolute Synthesis Rate (mg/g Tissue/d)
RNA Activity mg protein/mg RNA
- not significant, * $P<0.05$, ** $P<0.01$.

Dietary protein restriction resulted in a loss of liver protein during lactation such that the hepatic protein content (mg/g Tissue) of groups HL_2 and LL_2 were lower ($P<0.05$) than both that on day 1 of lactation (Table 3.4) and when compared to groups HH and LH. The hepatic RNA contents (mg RNA/g Tissue) for all lactation groups were lower ($P<0.05$) than those on day 1 of lactation, although they were not different from each other. Hepatic RNA activities were also not significantly different between the four lactation groups,

although the RNA activity of the HH group was significantly increased compared to day 1 ($P<0.05$) (Table 3.4).

Hepatic energy expenditure on day 13 of lactation did not differ between the groups and was also not different to that at the start of lactation.

Duodenal Mucosa: The rate of protein synthesis and tissue respiration of the duodenal mucosa for the four lactation treatment groups are shown in Table 3.9.

Table 3.9. Effect of gestation and lactation dietary treatments on the duodenal mucosa protein synthesis, composition and O_2 consumption (nmoles O_2/mg protein/min) on day 13 of lactation.

DIETARY SEQUENCE FROM DAY 12 GESTATION - DAY 13 LACTATION								
						DIET EFFECT		
	HH	LH	HL_2	LL_2	SD	GEST.	LACT.	INTER.
FSR (%/d)	150.4	127.0	140.2	156.9	31.5	-	-	-
ASR (mg/g/d)	169.7	136.9	151.0	155.6	31.9	-	-	-
Protein (mg/g)	113.12	109.10	107.93	99.99	11.8	-	-	-
RNA (mg/g)	6.72	6.63	6.71	6.33	0.8	-	-	-
RNA Activity	25.93	20.71	22.20	24.58	4.6	-	-	-
O_2 Consumption								
Total Respiration	5.2	3.6	4.0	4.0	1.4	-	-	-
Ouabain Insensitive	4.1	2.8	3.2	3.4	1.1	-	-	-
Ouabain Sensitive	1.1	0.8	0.8	0.6	0.8	-	-	-
% Inhibition	19.5	21.5	18.7	15.5	6.3	-	-	-

FSR Fractional Synthesis Rate
ASR Absolute Synthesis Rate (mg/g Tissue/d)
RNA Activity mg Protein/mg RNA
- not significant.

On day 13 of lactation the rate of mucosal protein synthesis and mucosal protein and RNA contents (mg/g tissue) were not significantly affected by dietary protein level and thus feed intake during lactation. Mucosal protein synthesis and composition were also, in general, not significantly altered between day 1 and 13 of lactation (Table 3.4), although the ASR (mg protein/g Tissue/day) of group HH was increased ($P<0.05$). The RNA activity on day 13 was not significantly affected by dietary protein content during lactation, although again the RNA activity of group HH was increased compared to day 1 ($P<0.05$).

In a similar way to mucosal protein synthesis, tissue respiration (nmoles O_2/mg protein/min) on day 13 of lactation was not significantly affected by dietary protein content and did not differ between day 1 and 13 (Table 3.9). On day 13 of lactation mucosal Na^+,K^+-ATPase activity accounted for between 15.5 - 21.5 % of total mucosal energy expenditure, which was comparable to that of the mammary gland and liver (Tables 3.7 and 3.8).

DISCUSSION

Previous research has shown that during lactation feeding of a diet low in protein quantity and/or quality results in a suppression of maternal feed intake and thus a reduction in milk production, although such females can attempt to sustain lactational performance by utilising endogenous reserves of protein and energy (Naismith *et al.* 1982, Chapter 2). When dietary protein is limiting, the extent of maternal protein reserve repletion at parturition has a significant impact on a female's ability to sustain lactation, with more protein available for mobilisation allowing an increased feed intake and improved lactational performance (Chapter 2, Mahan *et al.* 1975).

In this study the objective of the gestational dietary treatments was to establish at parturition two groups of females that had distinct differences in the extent of their protein reserves. By utilising data from this experiment and a similar experiment (E1, Chapter 2) for females slaughtered on day 1 of lactation, it has been shown that the low protein dietary treatment during the second half of gestation significantly reduced maternal carcass protein content (11%) and hence protein reserves on day 1 of lactation without significantly affecting the size of the adipose stores or organ weights and composition. It appears that females offered the 65 gCP/kg DM diet during gestation were able to prevent foetal growth retardation at the expense of their protein reserves.

During lactation the feeding of diet L_2 resulted in a significant suppression of feed intake and an increased loss of body weight by groups HL_2 and LL_2 compared to that of the two high protein groups. However the capacity of group HL_2 to mobilise more endogenous

protein allowed them to sustain a higher feed intake and hence lactational performance over that of group LL_2 during the first half of lactation. However this ability was limited and intakes and litter weight gains during the second half of lactation for both groups were similar, and it has been suggested that this could be due to the exhaustion of the maternal protein reserves in group HL_2 (Chapter 2).

The depletion of a female's labile protein reserve prior to lactation had no impact on the milk production of such females when they were offered an adequate supply of dietary protein (Chapter 2). In fact their lactational performance and feed intakes were similar to females offered the same diet that had not been previously protein depleted, while at the same time the protein depleted females were attempting to replenish their protein reserves. However in this study group LH had a significantly reduced feed intake and lactational performance when compared to the other high protein group, this difference being most apparent during the first six days of lactation. It is possible that the reduced protein and energy intake of group LH during the first six days may have been the result of a considerable mobilisation of endogenous adipose stores, which has been implicated in the suppression of intake of a low protein diet (Naismith *et al.* 1982). It is worth noting that on day 12 of lactation intakes of the high protein diet (31.3 and 30.9 g DM for HH and LH) were considerably lower than intakes of a similar diet recorded in the previous study, being 45.2 and 48.6 g/d for HH and LH respectively (Chapter 2). Since during gestation the intakes of both diet H and L were comparable to that of similar diets used in the earlier study (Chapter 2), it is apparent that the intake of diet H during lactation was prevented from reaching levels previously recorded, although the reasons behind this impairment are unknown. However, it is possible that the higher levels of dietary polyunsaturated fat used in the earlier study stimulated an improved intestinal absorption and thus feed intake (Sagher *et al.* 1991). The lower intakes in this study are reflected in a reduced lactational performance.

Dietary protein restriction during the second half of gestation resulted in a significant depletion of maternal protein reserves (Table 3.2), and this is reflected in the

significant reduction in the weight (18 %) and protein content (16 %) of the gastrocnemius muscle. Zartarian *et al.* (1980) reported that the feeding of a 7.5 % protein diet from day 12 - 20 of gestation promoted the use of muscle protein as an endogenous supply of amino acids for foetal development and resulted in a loss of 10 and 9 % of muscle weight and protein content respectively. The greater loss of muscle weight and protein content reported in this study can be attributed to the degree and length of the restriction imposed and that Zartarian *et al.* (1980) reported the protein loss from a combination of leg muscles, each of which may respond differently to protein restriction (Bryant *et al.* 1982).

The net loss of muscle protein during gestation was probably aided by the significant reduction in muscle protein synthesis (FSR, ASR) that was recorded on day 1 of lactation, although muscle protein synthesis responds very quickly to a reduction in protein supply (Millward *et al.* 1978). However in the protein depleted females rates of muscle protein degradation cannot be calculated, therefore the possibility that degradation was enhanced cannot be ruled out. Other workers have suggested that such a decline in muscle protein synthesis during the catabolic phase of gestation in protein restricted females rats may spare amino acids for the developing feto-placental unit (Mayel-Afshar *et al.* 1983). Although muscle respiration on day 1 was not significantly affected by the gestation treatment, the reduction in amino acid supply did tend to reduce the activity of the Na^+,K^+-ATPase enzyme ($P<0.10$). Since this enzyme is thought to be involved in nutrient uptake by tissues, amino acid supply to the muscle cell may have been impaired and associated with the reduction in muscle protein synthesis (Adeola *et al.* 1989, Vandenburgh *et al.* 1981).

Protein and energy restriction from the end of the first trimester of gestation in rats has been shown to result in a significant reduction in mammary gland hypertrophy and hyperplasia by day 1 of lactation (Rosso *et al.* 1981). In this study, because of the isocalorific nature of the diets used, feeding of diet L during gestation resulted in a restriction in protein supply only, and by day 1 of lactation there was no significant effect on mammary size, composition or rates of protein synthesis and respiration. If from Table 3.2 we use the

mammary gland weights reported, mammary ASR for the high and low groups were approximately 793 and 676 mg protein/d respectively. Thus during gestation the level of dietary protein restriction imposed appeared not to impair mammary development in preparation for lactation. The reduced lactational performance of group LH during the first half of lactation was therefore not the result of an impaired mammary development prior to lactation.

Protein restriction during gestation also had no significant effect on liver or mucosal metabolism on day 1 of lactation. Proteins synthesised by the liver include fixed proteins associated with liver metabolism and export proteins such as plasma albumin. The advantage of using the flooding dose technique for measuring protein synthesis is that it measures the rate of total liver protein synthesis including the export proteins.

From previous studies it has been shown that the rate of liver protein synthesis (%/d) is not particularly sensitive to reductions in protein quantity and/or quality during lactation (Sampson *et al.* 1984c, 1986) and that the absolute rate (ASR, mg/d) is only reduced following a reduction in liver weight and protein content (Jansen *et al.* 1986). A similar lack of sensitivity in hepatic protein synthesis to dietary protein restriction during lactation is reported here, with the rate of protein synthesis on day 13 being unchanged from that on day 1.

However under adequate nutrition lactation is normally associated with hypertrophy of the intestines, liver and mammary gland (Williamson 1980) which is due to the elevation of feed intake (Canas *et al.* 1982). It can be assumed therefore that in this study the feeding of the high protein diet during lactation would promote such liver hypertrophy and thus considerably increase hepatic protein synthesis (mg/d). Using liver weights (in brackets), from a similar experiment (Chapter 2), of females slaughtered on day 13 of lactation and offered similar dietary treatments as in this study, rates of liver ASR (mg/d) would have been approximately 4150 (22.7 g), 3737 (22.6 g), 2272 (14.1 g) and 2146 mg/d (13.5 g) for groups HH, LH, HL_2 and LL_2 respectively. These values are higher than published data due to the

relatively heavier liver weights used, but it can seen that during lactation hypertrophy of the liver would exaggerate the difference in hepatic protein synthesis between the high and low protein groups. The loss of liver protein during lactation in response to a reduced dietary protein supply was detected in this study and previous studies (Jansen *et al.* 1986, Sainz *et al* 1986b) and would have modified the total hepatic protein synthesis (mg/d) of groups HL_2 and LL_2.

It is generally accepted that the gastrointestinal tract makes a greater contribution to body protein turnover than the liver. In this study rates of protein synthesis in the duodenal mucosa were between 127 - 157 %/d and these are close to previously reported values for rat intestinal mucosa (McNurlan *et al.* 1979). On day 13 of lactation rates of mucosal protein synthesis appeared to be unaffected by dietary protein level and hence feed intake. However the increase in intake of the high protein diet during lactation would be associated with an increase in the size of intestinal mucosa (Lichtenberger *et al.* 1979), providing an increased surface area and digestive capacity of the gastrointestinal tract. The protein turnover associated with the intestinal mucosa would therefore be expected to be considerably increased during lactation, with also a sizeable difference between females offered the high or low protein diets.

Protein synthesis in the mammary gland is not subjected to diurnal variation (Sampson *et al.* 1984c) unlike lactose and lipid production (Williamson *et al.* 1984). The timing of the protein synthesis measurement would therefore not have had a dramatic effect on the values obtained in this study. The quantity of dietary protein offered during lactation had a significant impact on the rate of mammary protein synthesis, with both mammary FSR and ASR (mg/g tissue/d) in groups HH and LH being significantly greater than that of the two low protein groups and also compared to that on day 1 of lactation. Previous studies have shown a significant effect of protein quantity on mammary protein synthesis in rats, while improvements in protein quality at a similar level of protein quantity have a more dramatic effect (Jansen *et al.* 1986, Sampson *et al.* 1986). Using mammary weights (in brackets), from

a previous experiment (E1, Chapter 2), for females slaughtered on day 13 of lactation in groups HH and LH, the total rate of protein synthesis would have been 2960 (25.0 g) and 3178 mg/d (28.0 g) respectively.

In rodents the bulk of mammary development is complete by around day 3 of lactation (Griffith *et al.* 1961). The increase in mammary protein synthesis between day 1 and 13 of lactation, supported by the high protein diet, would be associated with an increase in cell number and cellular activity (Knight *et al.* 1982) with the increase in cellular activity being more important after mammary development was complete. Similarly although the low protein dietary treatment prevented mammary protein synthesis from increasing during lactation, there may have been a shift in the type of protein being synthesised from more structural protein on day 1 to more milk protein on day 13.

Although it has been widely recognised that reductions in the supply of protein quantity and/or quality to the mammary gland during lactation significantly impairs mammary protein synthesis and hence milk secretion, its influence on milk composition is varied, with some authors reporting a significant reduction in milk protein content (Crnic *et al.* 1978) while others report no particular effect on milk composition (Grigor *et. al.* 1985, 1987a) although in the latter studies protein restriction was only imposed during mid lactation. It is possible that in this study the extended period of protein restriction could have resulted in alteration in both milk protein yield and composition.

The phenomenon of milk protein degradation prior to secretion is now widely accepted and has been reported from both *in vivo* (Oddy *et al.* 1988) and *in vitro* (Hasan *et al.* 1982) studies. It has been suggested that about a third of milk proteins synthesised are degraded before they can be secreted. It is clear that from the measurements made in this study that it is not possible to partition the protein synthesised between cellular and milk proteins. Also the quantity of milk protein degradation that occurs prior to secretion could not be estimated. However excluding these factors, when considered for all groups, mammary

ASR (mg/g tissue/day) was strongly correlated with daily litter weight gain on day 12 of lactation (r^2=61 %, P<0.001).

It should be noted that measurements of protein synthesis on 2 days during lactation can only represent an index of metabolism at that time and cannot give information about possible changes between those two points. Although depletion of maternal protein reserves prior to lactation did not prevent the rate of mammary protein synthesis of group LH, or synthesis in the other tissues considered, from reaching the level of group HH it is possible that the significantly lower lactational performance and hence milk production during the first half of lactation would have been associated with a lower level of mammary protein synthesis. Likewise the ability of group HL_2 to out perform group LL_2 during the same period could also be associated with considerable differences in mammary protein synthesis.

The maintenance of tissue protein turnover is supported by a variety of cellular mechanisms, one of which is the membrane transport system Na^+,K^+-ATPase which plays an essential role in the transport of amino acids and sugars across cell membranes. Na^+,K^+-ATPase activity accounts for a major part of cellular energy expenditure and on day 13 of lactation in this study represented between 28 - 37 %, 20 -23 %, 17 - 21 % and 16 - 22 % of tissue respiration in the gastrocnemius muscle, mammary gland, liver and duodenal mucosa respectively.

The proportion of tissue energy expenditure associated with Na^+,K^+-ATPase activity has been reported to be significantly increased during lactation in the intestinal mucosa of cows from 35 % (dry) to 54 % (peak) and liver of sheep from 37% (dry) to 45% (peak yield) (McBride et al. 1984, 1985a). The results of this study tend not to support this, with tissue respiration and the proportion associated with Na^+,K^+-ATPase activity in the liver and mucosa on the whole remaining unchanged between day 1 and 13 of lactation. However the Na^+,K^+-ATPase activity in the mammary gland was significantly increased during lactation in the groups offered diet H, and this reflects the increased metabolic activity and protein synthesis in this tissue. It should be noted that although the tissue respiration per

gram were unchanged, the organ hypertrophy associated with lactation would result in a considerable increase in total and Na^+,K^+-ATPase dependent energy expenditure in tissues such as the liver and gut.

In this study the proportion of tissue respiration associated with Na^+,K^+-ATPase activity in the liver and duodenal mucosa (approximately 20%) were considerably lower than other published data for the liver (37 - 45%) and mucosa (28 - 61%) (McBride *et al.* 1985a, 1985b). The lower contribution of Na^+,K^+-ATPase activity to tissue respiration reported here may be attributed to the more complex incubation medium used in this study.

Dietary protein restriction during gestation and lactation significantly reduced the rate of muscle protein synthesis on day 1 and 13 of lactation and ultimately promoted a loss in muscle weight and protein content. Rates of muscle protein synthesis have been closely linked with the activity of Na^+,K^+-ATPase (Adeola *et al.* 1989, Vandenburgh *et al.* 1981) and protein restriction during lactation significantly reduced the proportion of tissue respiration associated with this enzyme. Muscle protein synthesis (mg/day) and Na^+,K^+-ATPase activity (nmoles O_2/day) were highly correlated (r^2= 48.3%, $P<0.001$, n=29), further supporting the close association of muscle protein synthesis and the activity of this membrane transport system.

Estimations on the contribution that Na^+,K^+-ATPase activity makes to muscle total respiration varies between studies from 22 - 25 % in growing pigs (Adeola *et al.* 1989), 40 % in growing calves (Gregg *et al.* 1982b) and 42 - 46 % in dry and lactating ewes respectively (Gregg *et al.* 1982c). These compare with 24 - 32 % and 28 - 37 % on day 1 and 13 of lactation respectively in this study and further reflects the importance this enzyme has in muscle energy expenditure.

Results of a previous study suggested that during lactation improvements in dietary protein supply to females previously protein depleted allowed them to acheive a lactational performance similar to that of other females offered the same diet, while at the same time attempting to replenish their depleted reserves (Chapter 2). In this current study the LH group

showed a significantly increased rate of muscle protein synthesis (3.4 - 4.8 %/d) and RNA activity during lactation, and this could represent part of the mechanism involved in protein replenishment.

In humans, laboratory animals and farm livestock the capacity of lactating females to utilise their endogenous reserves of protein in support of milk production is now widely accepted. Involved in this mobilisation of protein are changes in muscle protein turnover such that DEGRADATION > SYNTHESIS. Techniques involving infusions of radio labelled amino acids can be used to estimate protein synthesis in lactating and non lactating animals. However there are no direct estimates of protein degradation *in vivo* and it is normally estimated from calculations involving fractional rates of protein synthesis and differences in protein accretion.

The loss of muscle protein during lactation by groups HL_2 and LL_2 in response to protein restriction reflects the mobilisation of maternal protein reserves in support of lactation. The significant reduction in muscle protein synthesis in group HL_2 could represent a shift in protein turnover that ensures that degradation exceeds synthesis and net muscle protein loss occurs. The rate of muscle protein degradation of the HL_2 group during lactation can be calculated from the changes in muscle FSR and net loss of protein.

If it is assumed that the reduction in muscle protein synthesis occurs rapidly in response to changes in protein supply (Garlick *et al.* 1973, Millward *et al* 1983) and that the loss of muscle protein during lactation occurs at a steady rate (linear) (93 mg/12d, 7.78 mg/d), then the average rate of protein degradation (FDR) during lactation was 5.95 %/d. This calculated rate of degradation is considerably greater than the FSR on day 1 of lactation. It might be concluded therefore that protein loss from the gastrocnemius muscle during lactation in rats is the result of a change in both FSR and FDR which contrasts with suggestions from other studies concerning lactating animals (Bryant *et al.* 1982, Swick *et al.* 1977, Vincent *et al.* 1985).

It is possible that this calculation of FDR may have under estimated the true degradation rate since from earlier studies it has been suggested that the mobilisation of maternal protein occurs predominantly during the first half of lactation (Chapter 2). The measurements made in this study cannot detect such changes in protein turnover occurring during early lactation and thus further work on protein loss during this period is required.

The partitioning of available amino acids towards the mammary gland during lactation will be promoted by a homeorhetic shift in the hormonal milieu. Lactation is associated with hypoinsulinaemia (Williamson 1980) and a reduced sensitivity of adipose tissue (Burnol *et al.* 1987) and skeletal muscle in rats (Burnol *et al.* 1987) and sheep (Vernon *et al.* 1990) to this anabolic hormone. These changes favour reduced nutrient utilisation by skeletal muscle and possibly allow a drop in muscle protein synthesis as part of the mechanism involved in protein catabolism. Muscle protein loss may also be promoted by an increase in circulating glucocorticoids which actively induce a reduction in muscle protein synthesis (Millward *et al.* 1983) and may also stimulate protein degradation (Odedra *et al.* 1982). More important than the circulating levels of individual hormones will be the ratio between insulin:glucocorticoid (Buttery 1983) and this balance is proposed to be involved in controlling protein catabolism during pregnancy (Naismith 1966).

Swick *et al.* (1977) suggested that labile protein reserves are not composed of a specific storage polypeptide and that in muscle no intracellular protein is immune to degradation. However, from several studies it has been shown that muscle protein fractions respond differently to dietary (Rikimaru *et al.* 1980) and anabolic stimuli (Adeola *et al.* 1992). Furthermore muscle protein breakdown ultimately involves the use of proteolytic enzymes present in the muscle cell. Proteinase enzymes include those associated with lysosomes and the calcium activated proteinase which has been associated with post mortem tenderisation of meat (Goll *et al.* 1983). To prevent unnecessary protein degradation the activity of these proteinases need to closely regulated. The fact that the calcium activated proteinase exists in an active and inactive form, is inhibited by an intracellular inhibitor and

their activity can be regulated by anabolic agents (Higgins *et al.* 1988), provides an opportunity for some control over degradation. Further work is required into the activity of the muscle proteinases during muscle protein loss in lactation and whether a particular protein fraction is targeted.

In summary it can be concluded that dietary protein restriction during lactation results in a significant reduction in milk secretion, although protein restricted females attempt to sustain lactation by mobilising endogenous reserves of protein with the extent of reserve repletion having a significant impact on this ability. The reduced milk secretion is reflected in an impaired mammary protein metabolism, although other tissues appear to be less sensitive to reductions in protein supply during lactation. The mobilisation of maternal protein reserves during lactation involves changes in both muscle protein synthesis and degradation. The reduction in muscle protein synthesis is also associated with reduced activity of the Na^+,K^+-ATPase enzyme.

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CHAPTER FOUR

EXPERIMENT E3

THE EFFECT of DIETARY PROTEIN RESTRICTION DURING LACTATION in RATS on the LOSS of MATERNAL PROTEIN, CHANGES in MAMMARY COMPOSITION and the ABILITY of LACTATING FEMALES to RESPOND to IMPROVEMENTS in DIETARY PROTEIN SUPPLY.

The Effect of Dietary Protein Restriction During Lactation in Rats on the Loss of Maternal Protein, Changes in Mammary Composition and the Ability of Lactating Females to Respond to Improvements in Dietary Protein Supply.

ABSTRACT

This study was undertaken to determine the rate of body protein loss in female rats subjected to protein undernutrition during lactation and whether a period of such undernutrition would affect a female's ability to respond to improvements in dietary protein supply. Following mating, multiparous female Sprague-Dawley rats were caged individually and offered a 215 g CP/kg DM diet *ad libitum* until parturition. Following parturition, an initial group of females (n=6) were culled for body composition and mammary gland analysis. The remaining females were then allocated to 3 groups; 2 were offered *ad libitum* access to a diet containing either 150 (H) or 90 (L) g CP/kg DM until day 12 of lactation, while the third group were offered diet L until day 5 and then diet H from day 6 until day 12 (L/H). Groups of females were then culled on day 3, 6, 9, and 12 of lactation for body composition analysis. Weight gain of a standardised litter was used as an indicator of lactational performance. By day 12 of lactation, all 3 groups had lost considerable amounts of body weight, although the feeding of diet L increased this ($P<0.05$) compared to diet H. Improvements in the supply of protein to group L/H reduced maternal weight loss ($P<0.05$) during the second half of lactation. Intake ($P<0.05$) and lactational performance ($P<0.001$) of group L were reduced compared to that of group H. Improvements in the protein supply to group L/H allowed a rapid improvement in feed intake and litter weight gain (day 6) to levels that were comparable to group H and greater than group L ($P<0.01$). On both diets considerable amounts of carcass protein were lost during lactation and diet L tended to increase this loss compared to diet H (7.59 vs 5.93 g), although not significantly ($P=0.059$). Day 12 carcass protein contents of group H and L were 42.84 and 40.44 g respectively, although this level of carcass protein was apparently reached before day 12 of lactation on diet L (day

9) at a calculated rate of loss of 1.01 g/d. Group H calculated rate of protein loss during lactation was 0.49 g/d. Body fat loss during lactation was even greater on both diets, being 20.0 and 24.3 g for H and L respectively. During the first half of lactation, mammary DNA content and cell number were unchanged in both group H and L. However, considerable mammary gland regression occurred as lactation progressed in both dietary groups ($P<0.05$), although this occurred earlier for group L (day 6 - 9) than for group H (day 9 - 12). These results suggest that rates of maternal protein loss can be varied in response to alterations in dietary protein supply, with the depletion of the body protein reserves occurring earlier the greater the rate of mobilisation. The period of protein undernutrition applied to group L/H did not prevent lactational performance from being increased when the protein supply was improved and this was aided by the maintenance of mammary integrity during the first half of lactation in group L.

INTRODUCTION

In non ruminants, information concerning rates of maternal protein catabolism during lactation is limited. It has been previously reported in this thesis that during a 12 day lactation, rats suckling a litter of 12 pups and offered a low protein/high energy diet catabolised 10.3 g of maternal protein (Chapter 2) which, assuming a linear rate of protein loss, approximates to 0.85 g/day. This compares with calculated rates of body protein catabolism reported by Kanto *et al.* (1980) of 0.68 g/d (12.9 g/21d), Naismith *et al.* (1982) of 0.48 g/d (5.8 g/14d) and Sainz *et al.* (1986b) of 0.59 g/d (4.13 g/7d). Variations in calculated rates of protein loss between studies may be associated with differences in the nutritional and lactational stresses imposed, the length of study period used and the source from which protein is lost. However, from changes in the weight gain of the standardised litter during lactation (Chapter 2) it was suggested that maternal protein reserves were curtailed sometime before the end of the study period and therefore the assumption that protein loss during lactation is linear underestimates the maximal rate of protein catabolism occurring *in vivo*.

The ability of farm livestock to respond to improvements in protein supply during established lactation has been widely reported. Abomasal infusions of casein illicit significant increases in milk yield and milk protein yield in cows and goats (Derrig *et al.* 1974, Oldham *et al.* 1984), with the greatest response being in females previously subjected to dietary restriction (Ranawana *et al.* 1977, Whitelaw *et al.* 1986). However, from studies with lactating sheep it seems that the extent of the protein restriction imposed significantly affects the females capacity to improve milk production when dietary protein supply is restored (Peart *et al.* 1970, Robinson *et al.* 1979).

In non ruminants, severe protein under-nutrition during gestation has no significant impact on a females lactational ability when adequate nutrition is provided immediately following parturition (Mahan *et al.* 1975, Chapter 2). However, whether lactating females subjected to severe protein restriction from parturition can respond to improvements in dietary protein supply, especially after their supply of endogenous protein is thought to be curtailed, remains uncertain.

Milk secretion by the mammary gland is clearly a function of the number and activity of mammary secretory cells (Wilde *et al.* 1989a). In both rodents and ruminants the increase in milk secretion to peak yield is associated with both increases in cell number and activity while the declining phase of lactation is primarily the result of a fall in cell number (Knight *et al.* 1982, Wilde *et al.* 1989a). From a number of studies in rats, the depression of milk production on day 15 of lactation, following feeding of diets low in protein quantity and/or quality, was thought to be associated with reductions in mammary mass, cellularity and cellular activity (Sampson *et al.* 1984a, 1984c, 1986), although mammary gland changes throughout lactation were not reported. How the mammary gland responds during lactation to alterations in it's supply of protein, both dietary and maternal, and how this reflects alterations in lactational performance will be reported in this study.

The objectives of the current study were to investigate the rate of protein loss in female rats subjected to protein under-nutrition during lactation, to determine whether such under-nutrition, and therefore protein depletion, affects a female's ability to respond to

improvements in dietary protein supply and how the mammary gland responds during lactation to such dietary treatments.

MATERIALS AND METHODS

Experimental Protocol

Sixty six multiparous female Sprague-Dawley rats (Harlan and Olac UK Ltd.) weighing on average 306.8 (± 3.0) g were caged individually in a room regulated at 22 °C, with relative humidity from 40 - 60 % and under a 12 hour light-dark cycle, with the light period from 0800 - 2000 hours. Starting with the heaviest individuals, females were placed in a wire bottomed cage with a proven male breeder for mating. The morning on which mating was confirmed, through the presence of vaginal plugs, was designated day 1 of gestation and the females were returned to solid bottomed plastic cages for the remainder of the experiment.

From day 1 of gestation all females were offered a high protein diet (H_G, 215 gCP/kg DM) (Table 4.1) *ad libitum* until parturition. Immediately following parturition an initial group of females (n=6) was selected for slaughter and used for carcass and mammary composition analysis (see below).

On day 1 of lactation the remaining females were allocated factorially to 3 groups; 2 were offered *ad libitum* access to a diet containing either 150 (H) or 90 gCP/kg DM (L) (Table 4.1) until day 12 of lactation, while the third group were offered diet L until day 5 and then diet H from day 6 until day 12 (L/H). Females (n=6) were slaughtered on days 3, 6, 9, and 12 of lactation for groups H and L and on day 12 for group L/H. Females slaughtered during lactation were used in carcass and mammary composition analysis (see below).

Table 4.1. Diet Formulation (g/kg DM)

	H_G	H	L
Casein[1]	215	150	90
Starch/Sucrose[2]	443	489	530
Corn Oil	192	211	230
Vitamin Mix[3]	50	50	50
Mineral Mix[3]	50	50	50
Corn Flour	43	43	43
Choline Chloride	7	7	7

[1] Casein supplemented with DL-Methioine (99%+1%)
[2] Starch and sucrose mixture in ratio 2 : 1
[3] Vitamin and mineral mix formulated to meet N.R.C. requirments 1978
Anti-oxidant (Butylated hydroxy toluene) : 0.001% Fresh Matter
Emulsifier (Lecithin) : 0.2% Fresh Matter
All diets were formulated to be isoenergetic with a constant carbohydrate : fat ratio of 2.31 : 1
Diet Analysis : Protein (g CP/kg DM) H_G 216; H 151; L 92
GE (MJ/kg DM) H_G 21.46; H 21.87; L 21.19

On day 1 of lactation, to standardise the lactational stress imposed, all litters were adjusted to contain 12 pups. Weight gain of this standard litter was recorded daily throughout lactation and used as a index of lactational performance. Dam body weights and feed intakes were recorded throughout the experiment, with dam feed intakes and litter weight gain during lactation being recorded at the end of each 12 hour photoperiod. Fresh feed was made available between 0800 - 1000 hours each day and the dietary change for group L/H occurred during this period on day 6. All females were given free access to drinking water.

Dams were killed by decapitation and the carcass, liver, mammary gland, gastrointestinal tract (empty) and viscera were dissected from all animals. Procedures involved in the analysis of carcass and mammary composition have been described elsewhere (Chapter 2, Appendix 1).

Mammary DNA was estimated using the method of Munro *et al.* (1969) with calf thymus DNA (Sigma) as standard. Mammary cell number was calculated using the equation developed by Winick *et al* (1965):

$$Cell\ Number\ (10^6) = \frac{mg\ DNA \times Organ\ Weight \times 1000}{6 \cdot 2}$$

where mg DNA represents mg DNA/g tissue and Organ weight represents total weight of mammary gland.

Statistical Analysis

The effect of dietary protein content on dam feed intake, litter weight gain and changes in maternal body weight were analysed using analysis of variance (Minitab). This applies to data both for the whole study period and for litter weight gain and feed intake on individual days. Analysis of variance was also used in the analysis of diet effects on the distribution of dam feed intake and litter weight gain between the two photoperiods, while within diet effects on this distribution were analysed using paired T-Test. The effects of diet and stage of lactation on mammary gland weight and composition were analysed using two way analysis of variance and the effects between diets on days and between days within diet were analysed using T-Test following calculation of least significant differences. Changes in maternal protein and fat contents with diet and stage of lactation were analysed using two way analysis of variance with body weight on day 1 of lactation as a covariate (Genstat5).

RESULTS

The maternal body weight changes, feed intakes, pup birth weights and litter size (pups/litter) following gestation of females offered diets H and L during lactation are shown in Table 4.2. There were no significant differences between Groups H and L in any of these criteria following parturition.

Table 4.2. Mean pup birth weight, maternal weight changes and feed intakes during gestation of dams offered diets H and L during lactation (Mean with SEM).

	GRAND MEAN	H	L
Body Weight D1 Gest.(g)	306.8 ± 3.0	308.4 ± 3.6	305.8 ± 4.5
Gestation Weight Gain[1](g)	57.1 ± 2.2	62.2 ± 3.4	55.7 ± 3.0
Gestation Intakes (g DM)	388.1 ± 5.8	398.9 ± 8.2	381.2 ± 8.1
Mean Pup Birth Weight (g)	6.6 ± 0.1	6.6 ± 0.1	6.5 ± 0.1
Litter Size (Pups/Litter)	12.8 ± 0.4	12.2 ± 0.7	13.3 ± 0.5

[1] Dam Weight Gain Following Parturition

The Effect of Lactational Dietary Treatment on Maternal Body Weight Changes, Feed Intakes and Litter Weight Gains During Lactation

The body weight changes, feed intakes and litter weight gains of dams slaughtered on day 12 of lactation and offered dietary treatments H, L and L/H are shown in Table 4.3.

Table 4.3. The effect of lactational dietary treatment on maternal weight loss, feed intakes and litter weight gains of females slaughtered on day 12 of lactation (Mean with SEM).

DIETARY TREATMENT	H (n=5)	L (n=6)	L/H (n=4)
Dam Weight Loss[1] (g)	59.5 ± 6.9[a]	85.6 ± 6.3[b]	44.0 ± 6.3[a]
Day 1 - 5 (g)	33.8 ± 7.1[a]	41.6 ± 4.9[a]	35.3 ± 5.1[a]
Day 6 - 12 (g)	25.7 ± 3.0[a]	44.0 ± 3.3[b]	8.7 ± 4.1[c]
Dam Feed Intake[1] (g DM)	252.0 ± 11.7[a]	196.7 ± 13.1[b]	269.6 ± 37.3[a]
Day 1 - 5 (g DM)	93.5 ± 7.6[a]	89.7 ± 7.1[a]	89.3 ± 19.1[a]
Day 6 - 12 (g DM)	158.5 ± 9.8[a]	107.0 ± 9.2[b]	180.3 ± 18·4[a]
Litter Weight Gain[1] (g)	168.0 ± 13.0[a]	91.4 ± 5.2[b]	145.4 ± 15.5[a]
Day 1 - 5 (g)	69.8 ± 6.9[a]	49.2 ± 2.7[b]	43.4 ± 7.4[b]
Day 6 - 12 (g)	98.2 ± 8.5[a]	42.2 ± 3.5[b]	102.0 ± 8.2[a]

[1] Data for 11 day study period
[a,b,c] Means with different superscripts differ significantly $P<0.05$

By day 12 of lactation all 3 groups had lost considerable amounts of body weight (Fig. 4.1). However the feeding of diet L led to a greater ($P<0.05$) weight loss when compared to that of diet H, especially from day 6 - 12 of lactation (Table 4.3). Improvements

in the supply of dietary protein to group L/H had a dramatic impact on maternal weight loss, being significantly lower from day 6 - 12 than groups H and L during this period (Table 4.3).

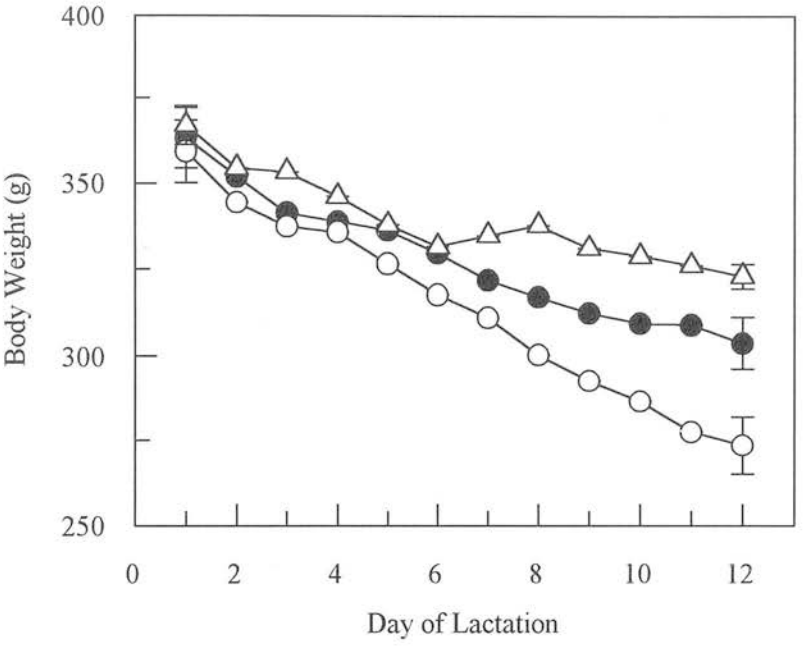

Fig. 4.1. Body weight changes of females offered dietary treatments H (●) (n=5), L (○) (n=6) and L/H (△) (n=4) between day 1 and 12 of lactation. Females were weighed at the same time each day and each point represents a mean with representative SEMs included for days 1 and 12.

The mean daily feed intakes (g DM/d) of all females offered diets H and L during lactation are shown in Fig. 4.2. When lactation intakes of all females used in this study are considered, the average intakes of diet H were significantly greater ($P<0.05$) throughout lactation than diet L, except for day 3. Intakes of diet H and L reached a peak around day 4 of lactation, after which no further increase was apparent. When diet H was offered on day 6 of lactation to females previously fed diet L, feed intake was rapidly and significantly increased, such that within 24 hours intakes of diet H by group L/H were comparable to group H and greater ($P<0.01$) than group L (Fig. 4.2). Intakes of groups H and L/H were not significantly different during the remainder of the experiment.

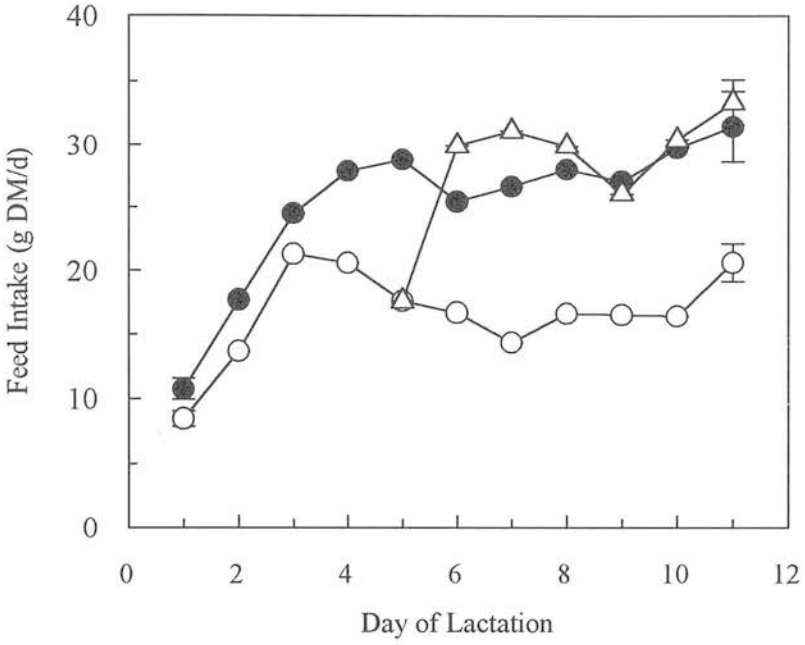

Fig. 4.2. Daily feed intake (g DM) of females offered dietary treatments H (●), L (○) and L/H (▲) during lactation. Data for all females offered diets H and L at each stage are included, with data for group L/H included in group L until day 6. Each point represents a mean and representative SEMs are included for days 1 and 11.

The greater supply of dietary protein received by group H, through an increased intake ($P<0.05$; Table 4.3) of a feed with a higher dietary protein content, allowed them to achieve a greater ($P<0.001$) litter weight gain during lactation compared to group L (Table 4.3). Increasing the protein content of the diet offered to females previously protein restricted until day 5 allowed them to promote litter growth from day 6 - 12 that was comparable to group H and greater ($P<0.001$) than group L. This improved supply of dietary protein was also able to compensate, in terms of total litter weight gain, for the feeding of diet L during the first 5 days of lactation (Table 4.3).

The greater total litter weight gain achieved by females offered diet H during lactation is reflected in their greater ($P<0.01$) daily litter weight gain throughout lactation (Fig. 4.3), when compared to females offered diet L. The rapid increase in dietary protein and energy intake by group L/H following the improvement in dietary protein content on day 6 of lactation (Fig. 4.2), promoted an immediate increase in their lactational performance (daily

litter weight gain). The litter weight gain achieved between day 6 and 7 of lactation was greater ($P<0.01$) than females offered diet L, while similar to that of females offered diet H and remained at this improved level for the rest of the experiment (Fig. 4.3).

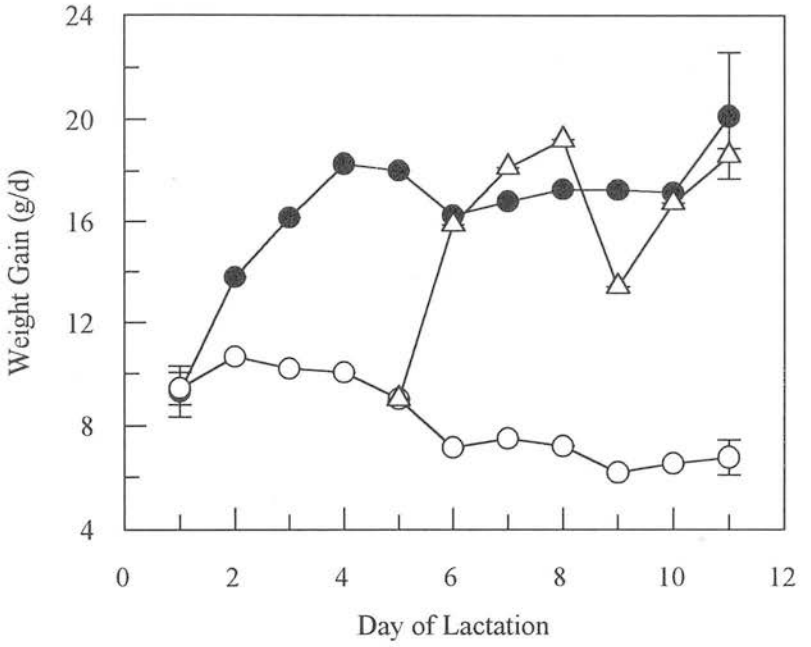

Fig. 4.3. Daily litter weight gain (g) of females offered dietary treatments H (●), L (○) and L/H (△) during lactation. Data for all females offered diets H and L at each stage are included, with data for group L/H included in group L until day 6. Each point represents a mean and representative SEMs are included for days 1 and 11.

The Proportion of Daily Litter Weight Gain and Feed Intake Associated With the 12 Hour Light Photoperiod

The proportions of daily litter weight gain and feed intake associated with the 12 hour light photoperiod are shown in Figs. 4.4 and 4.5.

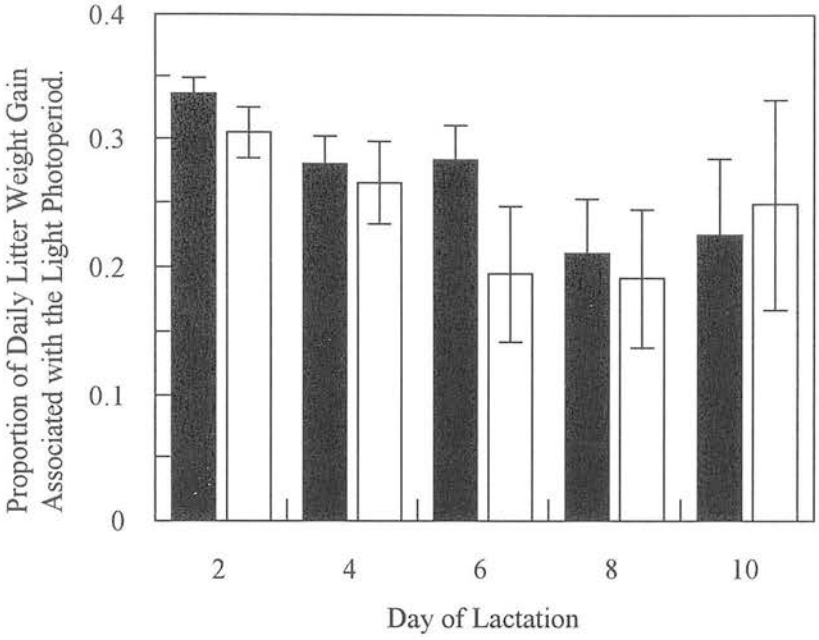

Fig. 4.4. The proportion of daily litter weight gain associated with the 12 hour light photoperiod of females offered diets H (■) and L (□) during lactation. Data for all females offered diet H and L (except group L/H) at each stage of lactation are included and bars represent a mean with SEM.

During lactation the dietary protein content had no significant effect on the distribution of daily feed intake and litter weight gain between the dark and light photoperiods (Figs. 4.4 & 4.5). However, for females receiving diets H and L there was a distinct diurnal pattern of both feed intake and litter weight gain, with intakes being more than 30% greater ($P<0.05$) during the dark photoperiod, and litter weight gains were more than 35% greater ($P<0.05$) than during the light photoperiod. The 12 hour light photoperiod was associated with only 30 - 40% and 20 -30% of feed intake and litter weight gain respectively.

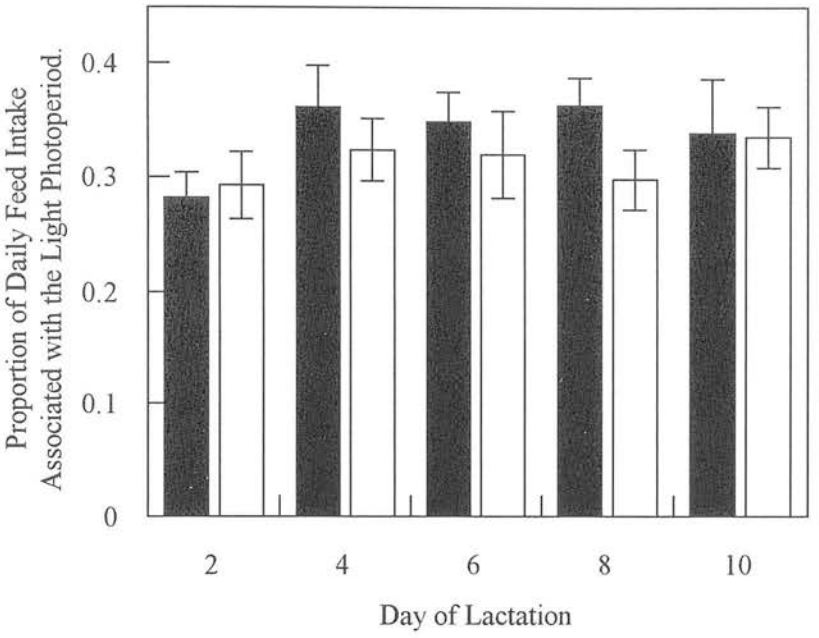

Fig. 4.5. The proportion of daily feed intake associated with the 12 hour light photoperiod of females offered diets H (■) and L (□) during lactation. Data for all females offered diet H and L (except group L/H) at each stage of lactation are included and bars represent a mean with SEM.

The Effect of Lactational Dietary Treatments on Changes in Maternal Body Composition During Lactation

Carcass Protein: Changes in the carcass protein content (adjusted for initial body weight on day 1 lactation) of females offered diets H and L during lactation are shown in Fig. 4.6.

At both levels of dietary protein there was a significant loss of carcass protein during lactation. However the females offered diet L tended, by day 12, to have lost more carcass protein (7.59 g) than those offered diet H (5.93 g), although by convention this was not significant at the 5% level (P=0.059). By day 12 of lactation diets H and L had reduced maternal carcass protein contents to 42.84 (± 0.52)g and 40.44 (± 0.71)g respectively (Fig. 4.6).

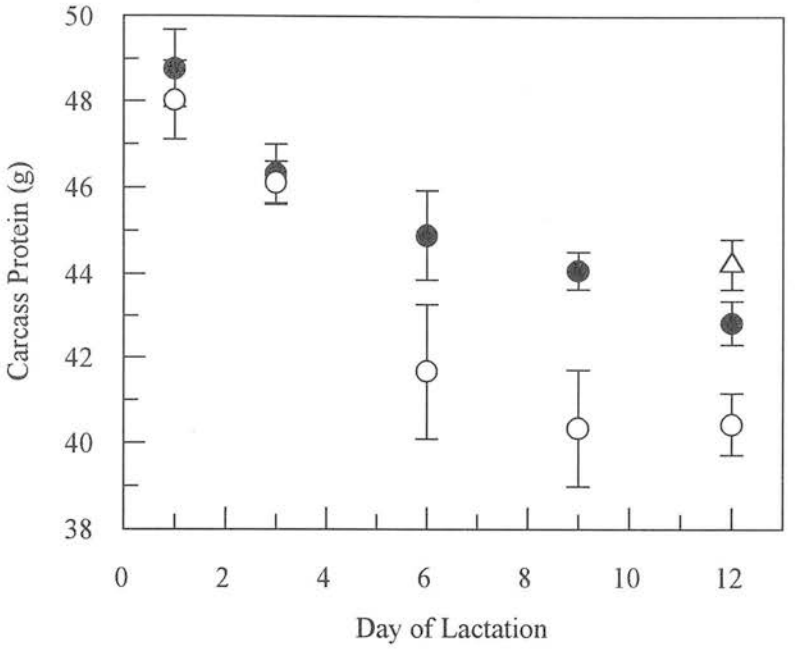

Fig. 4.6. Changes in the carcass protein content, adjusted for day 1 lactation body weight, of females offered diets H (●) and L (○) during lactation. Data are presented for groups of females slaughtered on either day1, 3, 6, 9 or 12 of lactation and individual points represent means with SEM. Data for group L/H (△) on day 12 are included for reference.

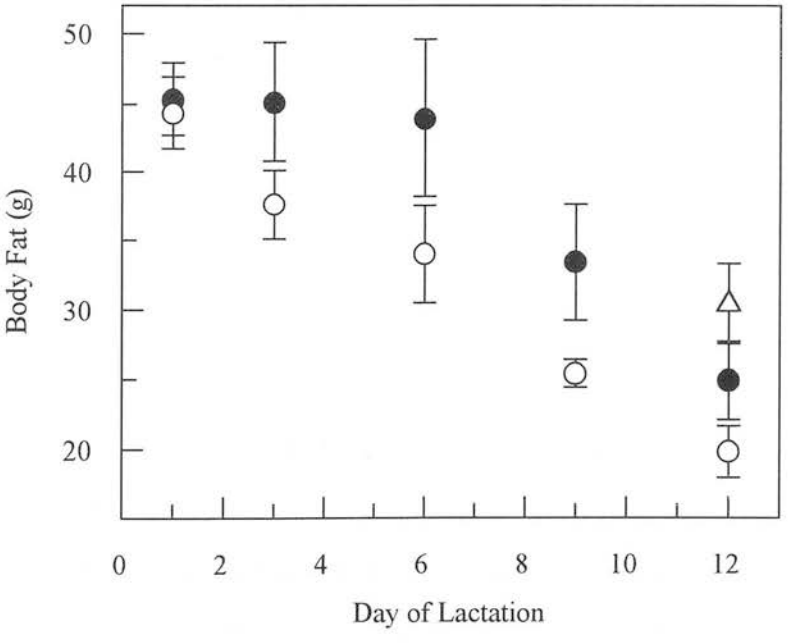

Fig. 4.7. Changes in the body fat content, adjusted for day 1 lactation body weight, of females offered diets H (●) and L (○) during lactation. Data are presented for groups of females slaughtered on either day 1, 3, 6, 9 or 12 of lactation and each point represents a mean with sem. Data for group L/H (△) on day 12 are included for reference.

Body Fat: Changes in maternal body fat contents (adjusted for day 1 lactation body weight) during lactation of females offered diets H and L are shown in Fig. 4.7.

Lactation was associated with a significant loss of body fat from females offered both levels of dietary protein. The feeding of diet L significantly increased body fat loss (24.3g) compared to that of diet H (20.0g) ($P<0.05$). The pattern of loss was also a little different, being linear for group L from day 1 of lactation while body fat mass in females of group H appeared to be maintained almost unchanged until day 6.

The Effect of Lactational Dietary Treatments on Mammary Tissue Composition and Cell Number

The changes in mammary composition during lactation of females offered diets H and L are shown in Table 4.4. The level of dietary protein offered to females during lactation had a significant impact on their mammary gland composition.

In females offered diet L, mammary gland weight declined during the course of lactation such that by day 9 and 12 of lactation their mammary weights were lower ($P<0.01$) than that of females offered diet H. Whilst the feeding of diet H from day 1 of lactation allowed females to maintain mammary gland size during lactation (Table 4.4), the feeding of diet H from day 6 of lactation to group L/H prevented the significant reduction in mammary gland size associated with the continued feeding of diet L.

Mammary dry weight displayed a similar pattern of response to dietary protein content during lactation as did mammary weight. One exception to this was that by day 12 of lactation mammary dry weight of females offered diet H was significantly lower than that on day 1 and also that of group L/H.

During the first six days of lactation, mammary protein content increased ($P<0.01$) in females offered both levels of dietary protein when compared to that on day 1. However females receiving diet L were unable to maintain this higher mammary protein content and by day 9 it had declined to a level similar to that on day 1 and lower ($P<0.05$) than that of

females offered diet H. Mammary protein content of group L/H on day 12 was similar to that of other females offered diet H.

Table 4.4. The effect of lactational dietary treatments on mammary gland weight and compositon (Mean with SEM).

DIETARY TREATMENTS	H	L	L/H
Weight[1] (g)			
Day 1*	21.7 ± 1.6^{w}	21.7 ± 1.6^{w}	
3	22.6 ± 1.6^{aw}	18.3 ± 2.2^{awX}	
6	19.9 ± 2.2^{aw}	16.5 ± 1.3^{axy}	
9	21.1 ± 2.1^{aw}	12.6 ± 0.7^{by}	
12	18.1 ± 1.0^{aw}	11.9 ± 1.1^{by}	21.1 ± 1.1^{a}
Dry Weight (g)			
Day 1*	10.6 ± 0.7^{w}	10.6 ± 0.7w	
3	10.1 ± 0.9^{aw}	7.7 ± 1.0^{ax}	
6	9.0 ± 1.1^{aw}	7.2 ± 0.9^{ax}	
9	8.7 ± 1.4^{aw}	4.7 ± 0.4^{by}	
12	6.1 ± 0.4^{ax}	4.3 ± 0.5^{by}	8.0 ± 0.5^{c}
Protein (g)			
Day 1*	1.6 ± 0.1^{w}	1.6 ± 0.1^{w}	
3	3.6 ± 0.2^{ax}	3.1 ± 0.1^{bx}	
6	3.5 ± 0.2^{ax}	2.8 ± 0.1^{bx}	
9	3.4 ± 0.2^{ax}	1.9 ± 0.1^{bw}	
12	2.6 ± 0.1^{ay}	1.7 ± 0.1^{bw}	2.9 ± 0.1^{a}
DNA (mg)			
Day 1*	59.5 ± 4.2^{w}	59.5 ± 4.2^{w}	
3	62.3 ± 7.6^{aw}	64.0 ± 5.7^{aw}	
6	69.3 ± 5.0^{aw}	62.8 ± 12.3^{aw}	
9	69.1 ± 7.0^{aw}	28.1 ± 3.9^{bx}	
12	38.7 ± 5.2^{abx}	26.7 ± 4.4^{bx}	57.4 ± 12.2^{a}

[1] Total mammary gland weight
* Mammary Composition of females slaughtered immediately following parturition
^{a,b,c} Means in the same row with different superscripts are significantly different $P<0.05$
^{w,x,y} Means in same column and block with different superscript are significantly different $P<0.05$

Changes in mammary cell number of females offered diets H and L during lactation are shown in Fig 4.8. In both dietary groups, mammary DNA content and cell number were not increased during early lactation and remained relatively unchanged until day 6. However, as lactation progressed there was a dramatic decrease in mammary cell number for both groups H and L ($P<0.05$), although this occurred earlier (between day 6 - 9) for group L than

it did for group H (between day 9 - 12). Improvements in the supply of dietary protein to group L/H allowed them to maintain a higher mammary cell number ($P<0.05$) on day 12 than females that continued to receive diet L.

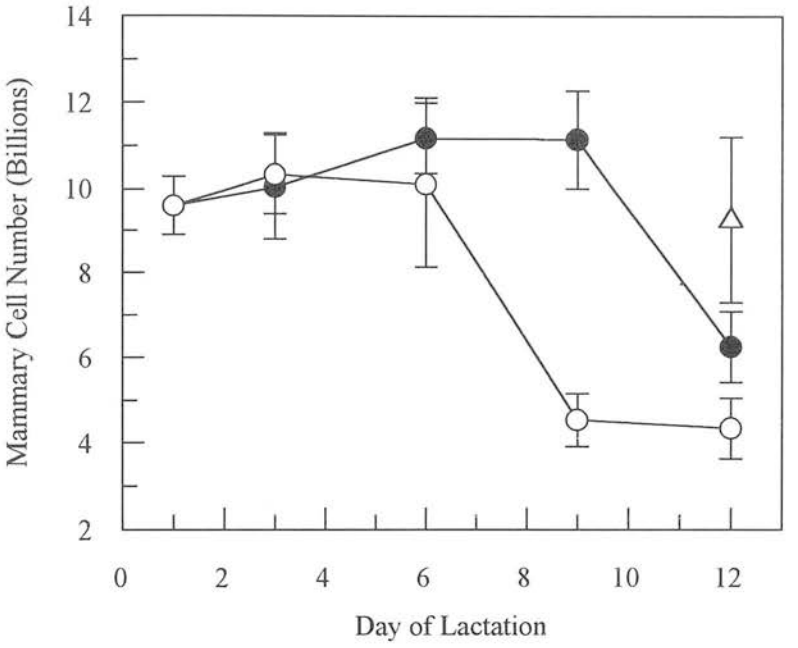

Fig. 4.8. Changes in the number (billions) of mammary cells for females offered diets H (●) and L (○) during lactation. Data are presented for groups of females slaughtered on either day 1, 3, 6, 9, or 12 of lactation and each point represents a mean with SEM. Data for group L/H (△) on day 12 are included for reference.

DISCUSSION

In most studies investigating the effects of dietary protein content on milk production in rats, levels of dietary protein were considered to be either adequate (high) or inadequate (low). Dietary protein levels considered inadequate for lactation include 10 % (Grigor *et al.* 1987a), 11 % (Naismith *et al.* 1982, Sampson *et al.* 1986) and 12 % (Sainz *et al.* 1986b) while adequate protein contents have ranged from 20 % (Grigor *et al.* 1987a) up to 36 % (Sainz *et al.* 1986b). To formulate the low protein diets, supplemented casein was replaced with carbohydrate which would ultimately reduce the diets energy density and therefore possibly have a positive effect on intake. Furthermore, recent studies by Friggens (1990) have

shown that the "adequacy" of the dietary protein level should also be considered in relation to the accompanying carbohydrate/fat content since the balance of nutrients of dietary and endogenous origin can have a dramatic impact on a females capacity to maintain milk secretion. In this thesis I have investigated maternal protein metabolism during lactation using isoenergetic diets of constant carbohydrate/fat ratio, that varied only in their protein:energy ratio, and this study extends my previous investigations by using an intermediate level of dietary protein not previously used in this laboratory.

Earlier studies have shown that lactating rats exhibit distinct diurnal patterns of feed intake and milk production, both being primarily associated with the dark phase (Grigor *et al.* 1987b, Munday *et al.* 1983). Grigor *et al.* (1987b) reported that these diurnal patterns can be disrupted by dietary protein restriction during established lactation. However the results of the current study do not support this, as females offered both levels of dietary protein showed distinct diurnal patterns of feed intake and litter weight gain throughout lactation (Figs. 4.4 & 4.5). Over 60% of daily feed intake and litter weight gain were associated with the dark period. Since females in this study had access to feed throughout the day, with fresh feed being made available each morning, the severe dietary protein restriction imposed resulted in reductions in only the quantity of food consumed and not the time when it was eaten. The reasons behind this difference in response to severe protein restriction are unknown. However, the significant disruption reported by Grigor *et al.* (1987b) may have resulted from the combined effects of using young virgin females, estimating diurnal feed intake on only 1 day in late lactation and imposing the dietary treatments during established lactation.

Gestation is often associated with a positive energy balance, with surplus energy-yielding nutrients being stored as fat which will ultimately be used to meet the increased nutrient demands of lactation (Naismith *et al.* 1982). The use of such energy reserves in support of lactation is not obligatory and depends on the size of the adipose stores available at parturition (Garnsworthy 1988, Rolls et al. 1984). The high protein/high energy diet offered to pregnant females in this study has been previously reported to facilitate the considerable accumulation of adipose stores during pregnancy (Chapter 2), and in this study body fat at

parturition totalled approximately 45 g. During lactation the catabolism of body fat occurred in females offered both levels of dietary protein, although to a significantly greater extent in females offered diet L (24.3 vs 20.0 g). This significant effect of dietary protein restriction on body fat mobilisation during lactation was not apparent in the earlier study (Chapter 2) and body fat was lost at approximately 2.84 g/d. In the current study, if the loss of maternal body fat during lactation is assumed to be linear, rates of loss can be calculated to be 1.92 and 2.18 g/d for groups H and L respectively (Fig. 4.7). Such a loss of body fat can supplement the lactating females supply of energy yielding nutrients by as much as 76 and 86 kJ GE/d respectively.

It is now well recognised that lactating females can utilise maternal reserves of protein as a buffer against dietary protein inadequacy during lactation (Lynch *et al.* 1988, Mullan *et al.* 1989b, Naismith *et al.* 1982), while the extent of reserve repletion at parturition has a significant impact on this ability (Shields *et al.* 1985, Chapter 2). The results of this study suggest that females offered high energy diets containing 90 and 150 g CP/kg are required to supplement this supply of dietary protein by mobilising their endogenous protein reserves. However the lower protein:energy ratio of diet L tended to increase the quantity of protein lost during lactation (7.59 vs 5.93 g), although by convention this was not significant at the 5% level (P=0.059).

From Fig. 4.6 it might be inferred that the dietary protein:energy ratio also influenced the rate at which maternal protein reserves were depleted. The carcass protein contents of dams offered diet H were found to decline throughout lactation to reach 42.84 (± 0.52)g on day 12, and the rate of protein loss during lactation was calculated to be 0.49 g/d. However in females offered diet L during lactation their carcass protein contents appeared to fall much more rapidly and reached a minimum on day 9 of 40.35 (± 0.71)g. The bulk of this protein loss had in fact occurred by day 6 (41.68 (± 0.60)g), with only minor changes in carcass protein content occurring during the remaining 6 days. Maternal protein reserves therefore appeared to be depleted more rapidly during early lactation by the high energy/low protein diet, L. Between day 1 and 9 of lactation the rate of protein loss was calculated to be

1.01 g/d, and is comparable with the rate of 1.19 g/d reported for dams offered restricted access to a 130 gCP/kg diet between day 7 and 14 of lactation (Sainz *et al.* 1986b). Despite this, the rate of maternal protein loss during lactation, as has been shown in earlier studies, occurs at a considerably reduced rate compared to that of body fat.

Although maternal protein reserves can have a buffering ability against reductions in protein supply during lactation, this capacity is constrained by the limited protein reserve available. Once these labile reserves are exhausted, milk production is totally dependent upon dietary protein supply. From a previous study using changes in pup growth as a qualitative indicator of milk production in rats, protein reserves were thought to be curtailed after six days of lactation in females offered a high energy, 90 g CP/kg diet (Chapter 2). The results of the current study support this conclusion; there does appear to be a metabolic limit to which maternal protein reserves can be depleted and in such protein restricted females this is reached between day 6 and 9 of lactation (Fig. 4.6).

The loss of carcass protein associated with the feeding of diet L during the lactation period (7.6g), represents only 15 % of day 1 carcass protein and is considerably less than the loss reported in an earlier study, (10.3 g/12d), involving lactating rats under a similar dietary treatment (Chapter 2). It is possible that the reduced protein loss estimated in this study resulted from differences in a lactating females ability to utilise her body protein reserves (Friggens 1990, Lynch *et al.* 1988) or unavoidable variations in the extent of reserve repletion at parturition due to an excessive catabolism of maternal protein in support of gestation (Naismith *et al.* 1976). Since in the current study no estimation of carcass protein changes during gestation were made, and that the dams used were slightly heavier and had a higher maternal protein mass at parturition, the impact of such a gestational protein loss on the extent of reserve repletion is difficult to quantify. However, if the maternal protein loss reported in the earlier study (Chapter 2) was achieved within 6/7 days, the rate of protein loss required would have been considerably greater (1.72 g/d) than that reported here (1.0 g/d).

In this study the feeding of diet H resulted in a significant catabolism of maternal protein, although apparently at a slower rate (0.49 g/d) than was promoted by diet L.

Previous studies have reported that lactating rodents do not draw upon muscle protein when an adequate supply of dietary protein, in excess of 20%, is offered from parturition (Millican *et al.* 1987, Naismith *et al.* 1982, Chapters 2 and 3). Although estimations of carcass protein before and after a 12 day lactation period (Chapter 2) could not confirm or reject the possibility that maternal protein is depleted and replenished during this period, a subsequent study has rejected this suggestion (Chapter 6). Therefore it appears that the level of dietary protein offered following parturition has a considerable influence on the amount and rate of protein loss that occurs during lactation.

Reductions in the quantity or quality of dietary protein offered to lactating rats results in a significant suppression of feed intake and therefore milk production (Grigor *et al.* 1987a, Jansen *et al.* 1986, Naismith *et al.* 1982, Sampson *et al.* 1984c). Reducing the dietary protein:energy ratio in this study (H vs L) had a similar effect on feed intake and lactational performance (Figs. 4.2 & 4.3), although females offered diet H were unable to achieve levels of litter weight gain (lactational performance) previously reported for similar females offered a diet of higher protein:energy ratio (215 g CP/kg DM) (Chapter 2).

During lactation females receiving diet H increased their daily feed intake, and therefore litter weight gain until day 4, after which no further increases were seen (Figs 4.2 & 4.3). This contrasts with the performance achieved by females offered a 215 g CP/kg DM isoenergetic diet. Although intakes, and litter weight gain, were similar on both diets until day 4, the females offered the 215 gCP/kg DM diet increased both their intake and lactational performance steadily for the remainder of lactation (Chapter 2). It has been suggested that the lower protein:energy ratio of diet H and the mobilisation of endogenous adipose stores during lactation combine to prevent feed intake from increasing further and therefore creating an imbalance of protein and energy yielding nutrients which cannot be disposed of via milk output, storage or oxidation (Friggens 1990).

Feed intakes and litter weight gains of females offered diet L during lactation were significantly reduced compared to those offered diet H, even though such females mobilised considerable quantities of endogenous protein and energy. During early lactation maternal

protein can make a considerable contribution to total protein supply (1.01 g/d), such that at peak feed intake (1.85 g protein/d) endogenous protein accounted for over 35 % of total protein supply. This compares with a contribution by maternal protein of only 10 % during the same period in females offered diet H. Although the supply of amino acids provided by endogenous protein breakdown may not match the precise requirements of the lactating mammary gland, it is clear that in such protein restricted females this endogenous supply of protein has a significant impact on milk production. Once the supply of maternal protein is removed milk production is dependent upon the intake and composition of dietary protein. In the current study this was reflected in an almost 40% reduction in litter weight gain during the latter stages of lactation.

The results of this and earlier studies in this thesis (Chapters 2 and 3) suggest that not only is the catabolism of maternal protein during lactation dependent upon the dietary protein:energy ratio, this also influences the rate at which such mobilisation occurs. Using isoenergetic diets (21.5 MJ GE/kg DM) that varied in only their protein content, it has been shown that while a high protein diet (215 gCP/kg DM) does not promote maternal protein utilisation, a 90 gCP/kg DM diet depletes the endogenous protein reserves at such a rate that the metabolic limit is reached within 6 - 9 days, after which little change in carcass protein occurs (Fig. 4.6). Between these two extremes lies the 150 gCP/kg DM diet used in the current study which also required the mobilisation of maternal protein to supplement dietary supply. However, although the quantity lost was determined by the extent of reserve repletion at parturition, the rate of this loss was only half that promoted by diet L.

During lactation, females that received diet H had their feed intake and lactational performance constrained around day 4, at which point the mobilisation of the maternal protein reserves supplied only 0.49 g/d of endogenous protein (10 % total supply) compared to the 1.01 g/d catabolised by females offered diet L. Although if lactation was allowed to continue it is likely that the feeding of diet H would have resulted in maternal protein being depleted to the level of group L (Fig. 4.6), if the additional endogenous protein that was available to group L during early lactation had been utilised by group H it could have alleviated, to some

extent, the imbalance of protein and energy yielding nutrients and possibly stimulated an improved lactational performance. However, this improved milk production would only be maintained until maternal protein reserves were exhausted.

At present, information on the mechanisms involved in controlling the loss of maternal protein during lactation is limited and the results of the current study suggest that further work into how such mechanisms equate the costs and benefits of regulating the rate of loss, and therefore limit the availability of maternal protein during early lactation, is required. However, it is apparent that a lactating female can recognise the balance of nutrients it receives via the digestive tract and in some way regulate her dependence upon endogenous protein reserves. Since it has been observed that the feed intake (DM) and litter growth during early lactation were comparable for dams offered the 150 and 215 gCP/kg DM feeds, it might be concluded that such a mechanism allowed dams offered diet H to adjust the supply of endogenous protein in response to the dietary protein content and litter demand, and therefore maintain litter growth at the level of the better fed females. Such an ability is further supported by the work of Friggens (1990) who showed that lactating females offered diets of constant protein content regulated the use of their endogenous protein reserves in response to variations in the dietary carbohydrate/fat ratio.

Whilst the lower rate of body protein loss in group H could be considered to have had a short term cost since feed intake and thus litter growth were constrained around day 4, the capacity to maintain a supply of endogenous protein throughout lactation may have long term biological benefits. Whereas the rapid rate of protein depletion in dams offered the severely imbalanced feed (L) is an essential short term buffer to the exogenous and endogenous nutrient supply, the slower rate of depletion when the dietary protein supply was moderately improved may provide females with the ability to maintain milk production until further improvements in dietary supply could be obtained. Whatever mechanism is involved in regulating the rate of maternal protein catabolism, it might be suggested that it attempts to integrate nutrient supply (diet) with nutrient demand (litter) and then balance the inadequacy through variations in the rate of protein mobilisation. Whilst from the current study it is

apparent that the rate of catabolism is influenced by the dietary protein content, the effect of variations in litter demand on such rates of loss are unknown and deserve further attention.

The depression in milk production associated with a period of protein undernutrition, either during established lactation or immediately following parturition, is thought capable of being quickly restored to its pre-restriction level provided the period of restriction is not excessive (Peart *et al.* 1970, Robinson *et al.* 1979). Although Robinson *et al.* (1979) reported that milk production in lactating sheep, following 7 days of protein restriction, was returned to its pre-restriction level when protein supply was restored, no indication was given as to the speed of the mammary glands response. In this study a period of severe protein restriction immediately following parturition did not prevent females of group L/H from increasing feed intake and lactational performance when dietary protein supply was improved. Their response was extremely rapid, and within 24 hours their intake and milk production was equivalent to females offered diet H throughout lactation. The fact that the intake of group L/H was similar to other females offered diet H during this period confirms the suggestion that there is a limit to the intake of a diet of this protein:energy ratio at this stage of lactation.

This rapid improvement in feed intake by group L/H females when offered diet H not only dramatically increased lactational performance but also practically stopped maternal body weight loss during the second half of lactation (Fig. 4.1). This reduction in weight loss was also reflected in an apparent alteration in the catabolism of maternal nutrient stores and on day 12 both carcass protein (Fig. 4.6) and body fat (Fig. 4.7) appeared to have been called upon less than in groups H and L. The mechanism by which this improvement in dietary protein altered the partitioning of available nutrients is uncertain, although the increased nutrient supply from day 6 may have adjusted the hormonal environment that controls tissue metabolism. A similar adjustment in nutrient partitioning has been suggested to occur in females protein depleted during gestation but offered adequate nutrition during lactation (Chapter 2).

The ability of group L/H to increase their lactational performance when nutrient supply was improved suggests that the period of protein restriction had not irreversibly

impaired mammary gland metabolism. Whether this ability would have been retained if the period of protein restriction had been extended, beyond the depletion of maternal protein reserves, remains uncertain.

Lactation is often associated with considerable hypertrophy of the liver, gastrointestinal tract, heart and mammary gland (Williamson 1980), while at the same time there is an expansion of cardiac output and blood flow to these tissues (Chatwin *et al.* 1969). In this study no such mammary gland hypertrophy was reported and in fact the feeding of diet L resulted in a significant loss of mammary weight during lactation, although the change in mammary composition (protein increasing at the expense of fat) reported in an earlier study (Chapter 2) was also evident here. Similar effects of protein and energy restriction on mammary gland size have been reported elsewhere (Grigor *et al.* 1987a, Jansen *et al.* 1986, Sampson *et al.* 1984c).

Mammary gland development in preparation for lactation in rats is thought to be complete by day 3 of lactation (Griffith *et al.* 1961), and following parturition the increase in milk production to peak yield is due both to the increase in cell number and cell activity (Knight *et al.* 1984). Mammary gland size (cell number) however was unchanged between day 1 and 6 of lactation in this study on both levels of dietary protein. It appears that either the levels of dietary protein used prevented further development of the gland or that development was complete at parturition. The increase in milk production to peak yield on both diets therefore appears to have been primarily associated with an increase in cellular activity.

Although mammary cell number appeared unchanged by dietary protein level during the early period of lactation, continued feeding of the 90 gCP/kg DM diet resulted in a significant fall in cell number after day 6. The reasons behind this significant mammary regression after day 6 are unclear. It is possible that the continued feeding of diet L exhausted the maternal protein reserves and therefore dramatically reduced the supply of protein to the gland. This reduced nutrient supply may then have ultimately stimulated cellular regression. The fall in milk production and therefore litter weight gain would reasonably be expected to be

associated with this fall in cell number. Whether the activity of the remaining cells would have been changed is uncertain.

The fact that the period of severe protein restriction during early lactation had no significant effect on mammary gland size (cell number) allowed group L/H to significantly increase their lactational performance when dietary protein supply was increased. Whether improvements in dietary protein supply after day 6, when mammary cell numbers were being significantly reduced, would have illicited a similar response remains in doubt. The lack of response to improvements in nutrient supply following extended periods of nutrient restriction may be associated with the same phenomenon (Peart *et al.* 1970, Robinson *et al.* 1979). However, since mammary integrity was maintained for longer in dams offered diet H (Table 4.4, Fig. 4.8), it might be suggested that improvements in lactational performance could be achieved later in lactation, when dietary protein was increased, than was possible with group L.

In summary it can be concluded that although reductions in the supply of dietary protein during early lactation significantly impair feed intake and milk production in rats, such restrictions do not prevent such females from improving their lactational performance when dietary protein supply is improved. Protein restricted females can mobilise their endogenous reserves of protein in an attempt to buffer the inadequacy of dietary protein supply, although the rate and amount of protein lost appears to be influenced by the level of dietary protein offered. Whether lactating females could respond to improvements in dietary protein supply following a prolonged period of protein restriction, especially after maternal protein reserves had become exhausted and considerable involution of the mammary cell population had occurred, however remains uncertain.

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CHAPTER FIVE

EXPERIMENT E4

THE EFFECTS of DIETARY PROTEIN RESTRICTION and STAGE of LACTATION on MILK COMPOSITION in RATS.

The Effects of Dietary Protein Restriction and Stage of Lactation on Milk Composition in Rats.

ABSTRACT

The effects of severe protein restriction following parturition on the changes in rat milk composition during lactation was investigated using multiparous female Sprague-Dawley rats caged individually following mating and offered a high protein diet (H; 215 gCP/kg DM) *ad libitum* until parturition. Following parturition, half the females continued to receive diet H, whilst the remainder were offered a diet low in protein (L; 90 gCP/kg DM) *ad libitum*. On days 2, 4, 8 and 12 of lactation groups of females from both dietary treatments were used to provide a milk sample. Milk samples were analysed for their lactose (enzymatically), protein (Coomassie Protein Assay), lipid (gravimetrically) and mineral contents (spectrophotometrically). The milk lactose content of group H increased with stage of lactation (r^2=0.85, P<0.001). Such an increase was prevented by diet L, and from day 8 of lactation the milk lactose of group L was lower (P<0.05) than in group H. Group H milk protein content did not change during lactation and averaged 90.7 mg/g. Dietary protein restriction reduced the milk protein content of group L such that on days 2, 4 and 12 of lactation it was lower (P<0.05) than group H. On day 8 of lactation the milk protein content of group L had increased (P<0.05) and was comparable to that of group H. For group H, milk lipid averaged 166.8 mg/g and was generally unchanged during lactation. Diet L increased (P<0.01) the milk lipid content (205.5 mg/g) compared to diet H and this was also significant on day 4 and 8 of lactation (P<0.05). Group L milk lipid content also increased between day 4 and 8 of lactation (P<0.05). Milk sodium declined during lactation in both dietary groups (P<0.01) but was unaffected by dietary treatment. Both milk calcium and phosphorus contents were increased (P<0.01) during lactation in both dietary groups, whilst protein restriction also increased the calcium and phosphorus content (P<0.05). Milk potassium and magnesium

contents were unaffected by dietary treatment or stage of lactation. This significant alteration in the milk composition of severely protein restricted dams, while possibly favouring the disposal of greater quantities of energy yielding nutrients, suggest that equations developed for the estimation of milk production in rats cannot be used under such conditions.

INTRODUCTION

In the modern U.K. dairy industry, not only are the volume and efficiency of milk production central to the economic survival of dairy farmers, the composition of the milk produced is also extremely important since the price paid per litre is determined by the milk's lactose, protein and fat contents (%). The effects of diet and nutrient supply on the composition of milk produced by the dairy cow has therefore received considerable attention, the results of which are described extensively elsewhere (Thomas *et al.* 1988).

However, unlike the milk produced by the dairy cow, which is used almost exclusively for human consumption, in most mammalian species milk remains the sole source of nutrients for the suckling young, whose growth is largely determined by the combination of milk quantity (yield) and quality (nutrient concentration), although milk yield is thought to be more important. Milk production by a lactating female is dependent upon the available nutrient supply and restrictions in the maternal intake of protein and energy yielding nutrients has been reported to significantly impair milk production in humans (Sampson *et al.* 1984a), cattle (Oldham *et al.* 1979), pigs (Mullan *et al.* 1989a) and rats (Naismith *et al.* 1982, Sampson et al. 1986). In addition to reducing milk yield, dietary protein restriction has been shown to have a significant impact on milk quality by reducing milk protein content in cattle (Oldham *et al.* 1978), humans (Forsum *et al.* 1980) and rats (Crnic *et al.* 1978, Sturman *et al.* 1986).

Despite the fact that the laboratory rat has been used extensively as a model in nutritional and biochemical studies of lactation, and that numerous experiments have provided

information on rat milk composition and the changes associated with lactation, the conditions that influence such changes during lactation are still confused. The majority of these studies have investigated either the changes in milk composition associated with stage of lactation and parity (Chalk *et al.* 1979, Keen *et al.* 1981, Luckey *et al.* 1954, Nicholas *et al.* 1981) or the effects of dietary protein restriction on milk composition at one sample point in lactation. Monitored in this way (single point sampling), protein restriction throughout both gestation and lactation (Sturman *et al.* 1986) or lactation only (Crnic *et al.* 1978, Grimble 1981) significantly lowered milk protein content, while protein restriction during established lactation reduced only the milk's whey protein content (Grigor *et al.* 1985, 1987a, 1989). The influence which severe protein under-nutrition has on the temporal patterns of milk composition throughout lactation remains to be elucidated.

It has already been reported in this thesis (Chapters 2 and 4) that reductions in the protein:energy ratio of diets offered to lactating rats results in a significant suppression of feed intake and impaired milk production, even though such females attempt to buffer this dietary protein inadequacy by mobilising their endogenous reserves of protein. Although this supply of endogenous protein cannot sustain milk production at the level of similar females offered adequate dietary protein, the extent of reserve repletion at parturition can have a significant impact on lactational performance. It is not known whether such protein restriction and maternal protein catabolism had any effect on milk composition as the relevant measurements were not made. However, changes in the weight gain of a standardised litter during mid lactation, a qualitative index of milk yield, suggested that maternal protein reserves had become depleted and constrained milk production by reducing the amino acid supply to the mammary gland (Chapters 2 and 4). Whether this reduced litter weight gain was the result of a diminished milk yield alone or whether there were associated changes in milk quality is uncertain. Since the concentrations of fat and protein in milk are determined largely by their rate of synthesis relative to that of lactose, and thus the flow of water into the golgi body

(Davies *et al.* 1983), milk protein production could have been impaired through the reduced protein supply and thus altered milk composition.

Using the weight gain of rat pups during lactation as an index of milk production has limited value because no consideration can be given to possible variations in milk composition. Milk yields from lactating dams can be estimated using a variety of other techniques, including the increase in mammary weight following the separation of dams and pups (Grigor *et al.* 1987b) or the dilution of tritiated water in rat pups (Knight *et al.* 1984). Such invasive and destructive techniques are technically laborious and therefore not practicable in many studies of lactation. However, the problem of accurately estimating milk yield during lactation in the rat has been aided by the development of techniques that estimate milk yield from the weight gain of rat pups while accounting also for their maintenance requirements (Grigor *et al.* 1987a, Sampson *et al.* 1984b). Although these techniques were developed using well nourished dams during the first two weeks of lactation, they are thought to be equally applicable to undernourished dams provided the dietary restriction does not alter milk composition. In order to evaluate the degree of inaccuracy which might be associated with the assumption of a constant milk composition in lactational studies with rodents, this study has explored the impact of dietary protein restriction on milk composition.

The objective of the current study was to investigate the effect of severe protein restriction following parturition on the changes in rat milk composition during lactation.

MATERIALS and METHODS

Experimental Protocol

Thirty-two multiparous female Sprague-Dawley rats (Harlan and Olac UK Ltd.) weighing on average 306.7 (± 4.9)g were caged individually in a room regulated at 22 °C, with relative humidity from 40 - 60% and under a 12 hour light dark cycle, with the light period from 1100 - 2300 hours. Starting with the heaviest individuals, females were placed individually in a wire bottomed cage with a proven male breeder for mating. The morning on

which mating was confirmed, through the presence of vaginal plugs, was designated day 1 of gestation and the females were returned to solid bottomed plastic cages for the remainder of the experiment.

From day 1 of gestation all females were offered a high protein diet (H, 215 gCP/kg DM) (Table 5.1) *ad libitum* until parturition. The day on which parturition occurred was designated day 1 of lactation.

Table 5.1. Diet formulation (g/kg DM).

	HIGH (H)	LOW (L)
Casein[1]	215	90
Starch/Sucrose[2]	443	530
Corn Oil	192	230
Vitamin Mix[3]	50	50
Mineral Mix[3]	50	50
Corn Flour	43	43
Choline Chloride	7	7

[1] Casein supplemented with DL - Methionine (99% + 1%)
[2] Starch and sucrose mixture in ratio 2:1
[3] Vitamin and mineral mix formulated to meet N.R.C. requirements 1978
Anti-oxidant (Butylated hydroxy toluene): 0.001% Fresh Matter
Lecithin (Emulsifier): 0.2% Fresh Matter
Diet Analysis : Protein (g CP/kg DM); H 21.40, L 9.40
GE (MJ/kg DM); H 21.59, L 21.45

Subsequently, from day 1 of lactation half the females continued to receive diet H *ad libitum* whilst the remainder were offered a low protein diet L (90 gCP/kg DM) (Table 5.1) *ad libitum*. During lactation, groups of females (n=4) from both dietary treatments were used to provide milk samples on day 2, 4, 8 and 12. Females were first separated from their litters for 2 hours at the start of the light cycle, with dams being allowed access to the appropriate diet during the separation period, after which time they were lightly anaesthetised (diethyl ether) and injected subcutaneously with 5 i.u. of oxytocin (Sigma) in saline. Milk samples (0.5 - 0.75 ml) were then obtained by gently stripping the left thoracic and abdominal teats and stored at - 20 °C prior to analysis. Individual females provided only one milk sample, and following milking the females were used for the analysis of tissue protein metabolism (which

will be reported separately, Chapter 6) and culled. The mammary gland from the side that had not been milked was also weighed and analysed for protein (Lowry *et al.* 1951).

The diets used in this study were formulated to provide approximately 21 MJ GE/kg DM with a constant carbohydrate:fat ratio of 2.3:1 (Table 5.1). In order to maximise the lactational stress imposed, all litters were standardised to 12 pups on day 1 of lactation and litter weights were recorded daily throughout lactation. Dam body weights and feed intakes were also recorded throughout the experiment. All females were given free access to drinking water.

Litters from all females were analysed for their protein and fat contents as previously described (Chapter 2, Appendix 1).

Analysis of Milk Composition

All milk samples were analysed individually for their lactose, total protein, total lipid and mineral contents (Appendix 1). Milk lactose content was estimated enzymatically using a lactose test kit (Boehringer Manneheim 176303) after the milk sample had been deproteinised using trichloroacetic acid. Total milk protein was measured through reaction with the Coomassie Protein Assay Reagent (Pierce Chemical Company) using casein as a standard, while total milk lipid was estimated gravimetrically (Bligh *et al.* 1959) following a three times extraction with chloroform-methanol (2:1 v/v). The milk samples were also assayed for sodium, potassium, calcium, phosphorus and magnesium using an Inductively Coupled Plasma Spectrophotometer (Jorrell ICAP 61E).

Statistical Analysis

Dietary effects on maternal weight loss, feed intake during lactation and litter weight gains were assessed by analysis of variance. The effects of diet and stage of lactation on milk composition were analysed using two-way analysis of variance, and by calculations of least

significant differences T tests were used to compare means of samples between individual days and diet.

RESULTS

The maternal body weight changes, feed intakes during gestation, litter size (pups/litter) and mean pup birth weight following parturition of females subsequently offered diets H and L during lactation are shown in Table 5.2. Females offered diets H or L during lactation had achieved similar levels of maternal weight gain, feed intake and litter size during gestation.

Table 5.2. Maternal body weight changes, feed intakes during gestation and litter size following parturition of dams subsequently offered diets H or L during lactation (Mean with SEM).

LACTATION DIET	H (n=16)	L (n=16)
Dam Weight D1 Gest (g)	310.1 ± 5.4	298.4 ± 5.1
Dam Weight Gain (g)	48.4 ± 4.0	56.0 ± 3.9
Feed Intake (g DM)	350.6 ± 9.4	368.7 ± 9.0
Litter Size (pups/Litter)	9.5 ± 1.0	11.0 ± 1.0
Mean Pup Birth Weight (g)	6.4 ± 0.1	6.5 ± 0.1

Effects of Lactational Dietary Treatment on Maternal Body Weight Changes, Feed Intakes, Litter Weight Gains and Mammary Gland Weight and Protein Content During Lactation

The feed intakes, body weight changes and litter weight gains of dams offered diet H or L during lactation and slaughtered on day 12 are shown in Table 5.3.

The changes in maternal body weight, feed intakes and lactational performance were all significantly affected by the lactational dietary treatments. The feeding of diet L during lactation resulted in a considerably greater weight loss by group L ($P<0.001$), which was also greater than their weight gain during gestation (Table 5.2).

The mean daily feed intake (g DM/d) and litter weight gain (g/d) of all females offered diets H and L during lactation are shown in Figs. 5.1 & 5.2. Except for day 1, both

feed intake and litter weight gain were greater throughout lactation for females offered diet H ($P<0.05$) and reached a maximum of 43.0 (± 4.1) g DM/d and 31.2 (± 1.5) g/d respectively on day 11. Consequently, this resulted in a greater total feed intake (g/DM) and litter weight gain (g) during lactation for females offered diet H ($P<0.01$) (Table 5.3).

Table 5.3. Effect of lactational dietary treatments on maternal weight loss, feed intake, litter weight gain, mammary gland weight and protein content during lactation (Mean with SEM).

TREATMENT GROUP		H			L	
Dam Weight Loss (g/12d)[1]		12.5	±	6.8	92.9	± 9.0***
Dam Feed Intake (g DM/12d)[1]		342.4	±	17.8	187.5	± 15.6**
Litter Weight Gain (g /12d)[1]		245.8	±	14.7	86.9	± 4.7**
Mammary Weight[2] (g) :	Day 2	9.03	±	0.87	10.70	± 1.10
	4	10.17	±	1.54	8.57	± 1.36
	8	12.28	±	0.96	8.28	± 0.58*
	12	13.63	±	1.27	7.36	± 0.27*
Mammary Protein (g) :	Day 2	0.89	±	0.08	1.12	± 0.16
	4	1.08	±	0.19	0.92	± 0.15
	8	1.33	±	0.13	0.75	± 0.06*
	12	1.39	±	0.06	0.69	± 0.03*

DM Dry Matter
[1] Means of Dams Slaughtered on day 12
[2] Right hand abdominal and thoracic glands
*** $P<0.001$, ** $P<0.01$, *$P<0.05$

The feeding of diet H during lactation promoted a significant increase in mammary gland weight and protein content, whilst the low protein diet resulted in significant mammary regression (Table 5.3). Consequently, on days 8 and 12 of lactation both mammary gland weight and protein content were higher in dams offered diet H ($P<0.05$).

The changes in litter protein and fat contents during lactation on both dietary treatments are shown in Fig. 5.3. Both the litter protein and fat contents of group L were lower ($P<0.01$) than those of females offered diet H during lactation, with this difference becoming significant after day 4.

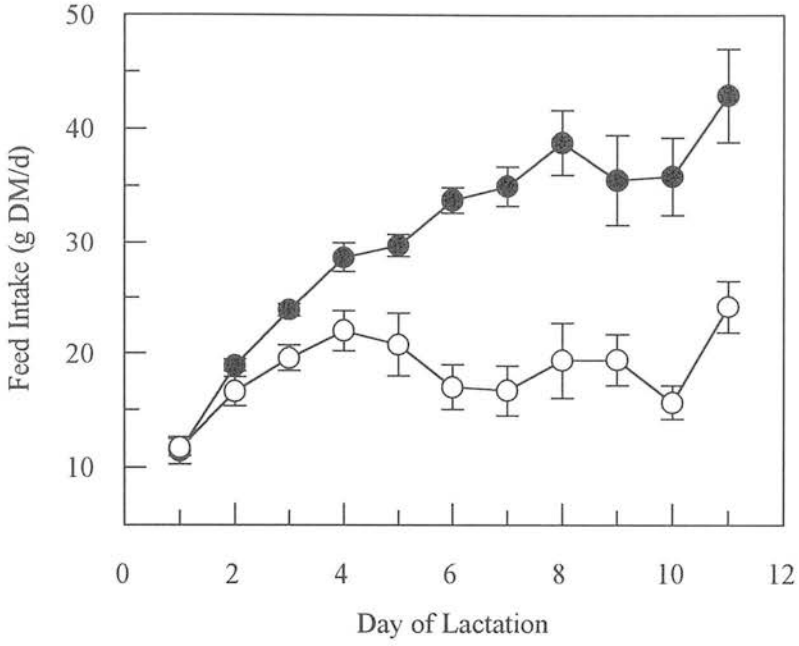

Fig. 5.1. Daily feed intake (g DM) of females offered diet H (●) or L (○) during lactation. Data for all females offered diet H and L are included and each point represents a mean with SEM.

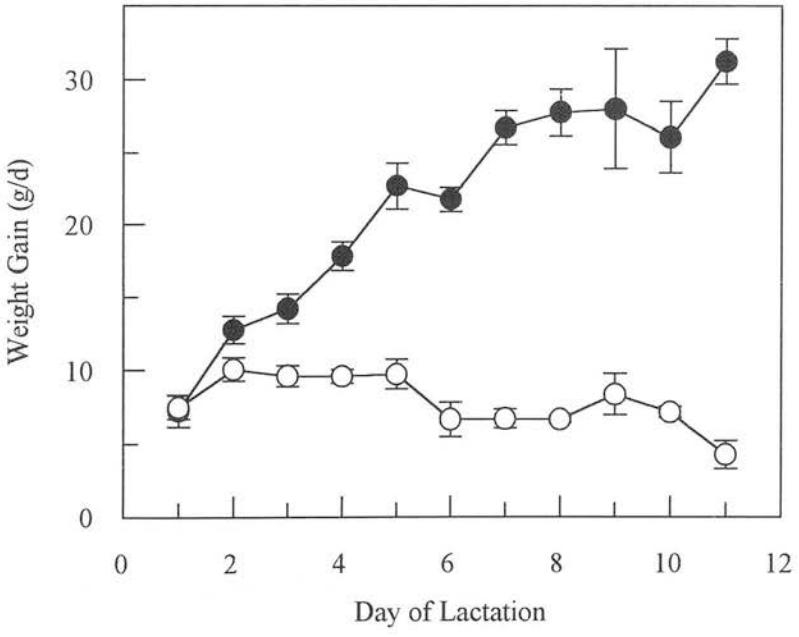

Fig. 5.2. Daily litter weight gain (g) for females offered diet H (●) and L (○) during lactation. Data for all females offered diet H and L are included and each point represents a mean with SEM.

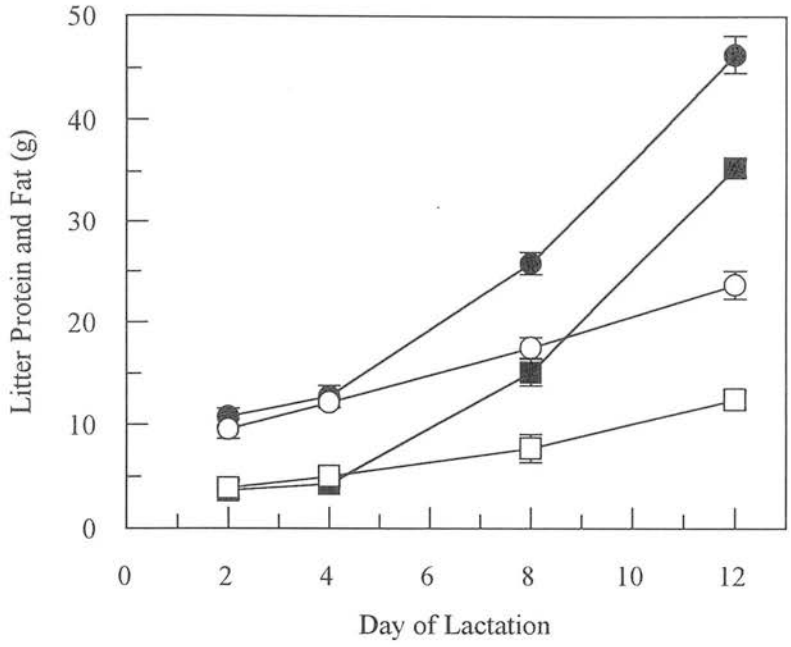

Fig. 5.3. Changes in the litter content of protein (●) and fat (■) for females offered diet H (●) and L (○) during lactation. Data are presented for standard litters (n=4) of 12 pups culled on either day 2, 4, 8, or 12 of lactation and each point represents a mean with SEM.

Effect of Lactational Dietary Treatments and Stage of Lactation on the Macro-nutrient Composition of Rat Milk

Lactose: Milk lactose content was significantly affected by both the dietary protein content and stage of lactation (Fig 5.4). Milk lactose of group H increased significantly with stage of lactation ($r^2 = 0.85$, $P<0.001$) whilst for group L it did not. By day 8, milk lactose content was significantly greater for group H than group L ($P<0.05$) and remained so on day 12.

Total Protein: The effects of lactational dietary treatment and stage of lactation on milk protein content are shown in Fig. 5.5. During the period of lactation studied, the milk protein content of group H did not change significantly and averaged 90.7 (± 1.9)mg/g.

The feeding of diet L during lactation resulted in the milk protein content on day 2 (79.8 (± 2.6) mg/g) and 4 (76.4 (± 0.7) mg/g) being lower ($P<0.05$) than those of group H (90.5 (± 4.1) and 89.4 (± 4.7) mg/g). However, by day 8 the milk protein content of group L had increased ($P<0.05$) to 98.3 (± 3.4) mg/g, which did not differ from that of group H (91.3

(± 4.0) mg/g). Milk protein content of group L was not maintained at this level and by day 12 had fallen ($P<0.05$) to 81.2 (± 3.8) mg/g, and was again lower than that of group H ($P<0.05$).

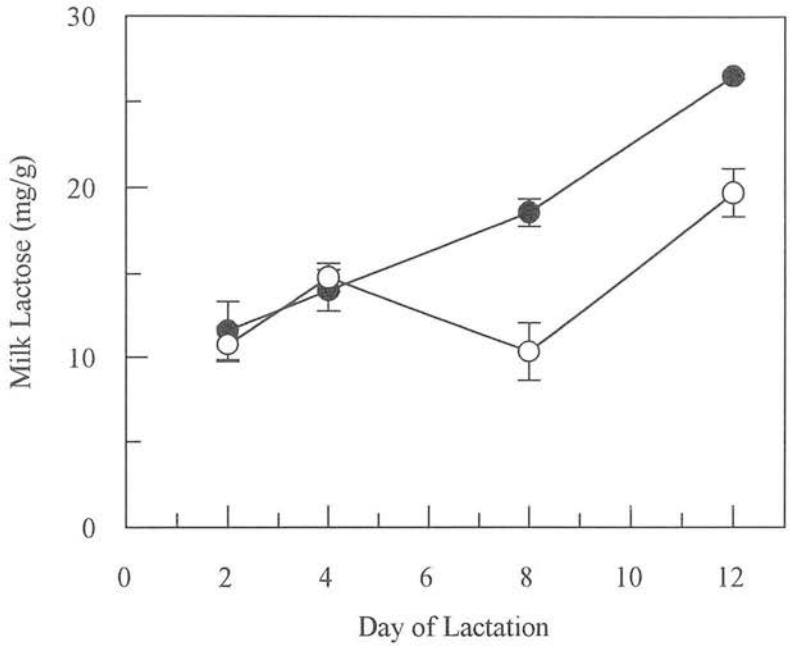

Fig. 5.4. Changes in the milk lactose content (mg/g) of females offered diet H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

Total Lipid: The effects of lactational dietary treatment and stage of lactation on milk lipid content are shown in Fig. 5.6. For group H, total milk lipid content averaged 166.8 (± 5.8) mg/g and, apart from the day 4 content being lower than that on day 8 ($P<0.05$), was not significantly changed during lactation. The feeding of diet L during lactation increased ($P<0.01$) the milk fat content to an average of 205.5 (± 13.5) mg/g.

By day 4 of lactation, the milk lipid content of group L (182.8 (± 8.5) mg/g) was higher ($P<0.05$) than that of group H (147.3 (± 10.3) mg/g). Group L milk lipid had increased further ($P<0.05$) by day 8 to 255.4 (± 14.8) mg/g and was again higher than that of group H ($P<0.001$). However, in a similar fashion to milk protein, the milk lipid content of

group L subsequently declined ($P<0.05$) and by day 12 there was no significant difference in milk lipid content between groups H (177.7 (± 7.9) mg/g) and L (201.3 (± 13.5) mg/g).

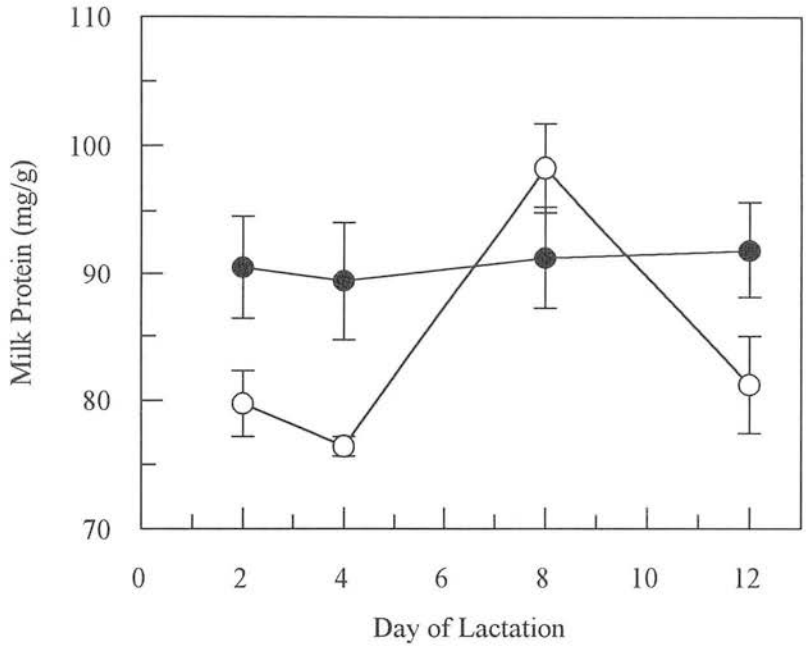

Fig. 5.5. Changes in the milk protein content (mg/g) of females offered diet H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

The Effect of Lactational Dietary Treatments and Stage of Lactation on the Sodium, Potassium, Calcium, Phosphorus and Magnesium Contents of Rat Milk

The procedure used for assaying the mineral content of the rat milk samples provided information on the total quantity of sodium, potassium, calcium, phosphorus and magnesium. The assay was unable to partition the ions between their diffusible and non-diffusible pools. The results reported can therefore only represent the total ion content of rat milk.

The effects of the lactational dietary treatments and stage of lactation on milk sodium content are shown in Fig. 5.7. Although the level of dietary protein had no significant effect, milk sodium content declined during lactation in both dietary groups ($P<0.01$).

Both milk calcium and phosphorus were significantly influenced by dietary protein content and stage of lactation (Fig. 5.8). Although on each day studied milk calcium contents were not significantly different, throughout lactation the low protein diet increased the milk calcium content of group L (2.77 (± 0.17) mg/g) when compared to group H (2.36 (± 0.15) mg/g) ($P<0.05$). Likewise, milk phosphorus content was increased in group L (2.48 (± 0.12) mg/g) when compared to group H (2.11 (± 0.11) mg/g) ($P<0.05$). Milk calcium content also increased through lactation ($P<0.01$) for groups H and L to values on day 12 of 2.86 (± 0.21) and 3.42 (± 0.34) mg/g respectively. The increase in milk phosphorus contents during lactation ($P<0.01$) reached a peak of 2.53 (± 0.11) and 2.79 (± 0.28) mg/g on day 8 for groups H and L respectively.

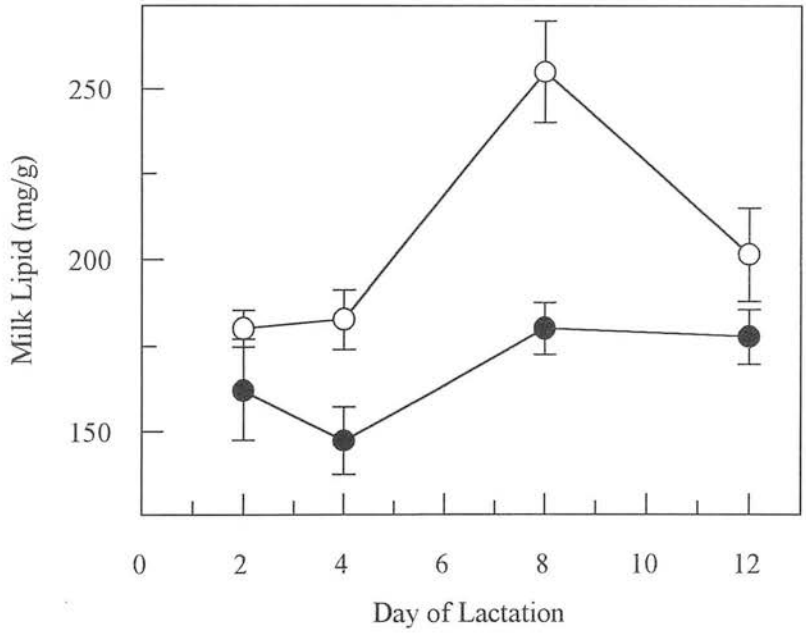

Fig. 5.6. Changes in the milk lipid content (mg/g) of females offered diet H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

Dietary treatment or stage of lactation had no significant effect on the milk content of potassium or magnesium. Potassium contents for groups H and L averaged 1.88 (± 0.07)

and 1.81 (± 0.01) mg/g respectively, while milk magnesium levels were 0.21 (± 0.01) and 0.22 (± 0.10) mg/g respectively.

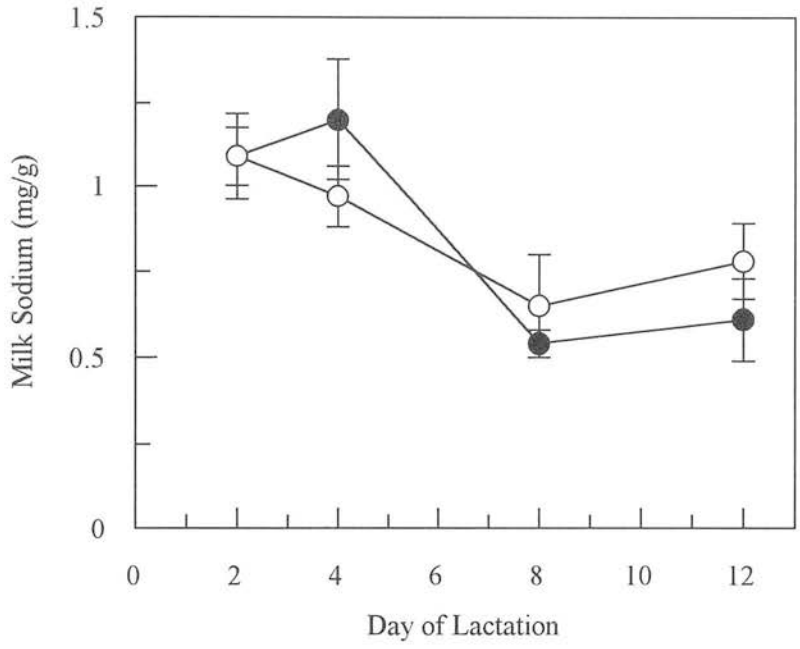

Fig. 5.7. Changes in the milk sodium content (mg/g) of females offered diets H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

Since milk salts exist primarily as either ions in solution, bound to protein or associated with casein micelles, they are concentrated in the milk's aqueous phase. Variations in the milk lipid content will therefore dilute the ion concentration in whole milk and possibly mask any changes in ion concentration associated with the aqueous phase. It is therefore worth considering the possible effects of dietary protein content and stage of lactation on the ion concentration of fat free milk (FFM).

The effects of dietary protein content and stage of lactation on the combined sodium and potassium contents (NaK) of FFM are shown in Fig. 5.9. The NaK content decreased with stage of lactation in groups H and L ($P<0.05$) to values on day 12 of 2.72 (± 0.03) and 2.94 (± 0.08) mg/g FFM respectively. Dietary protein content had no significant affect on the

NaK content of FFM and the decline during lactation was the result of a fall milk sodium content (Fig. 5.7).

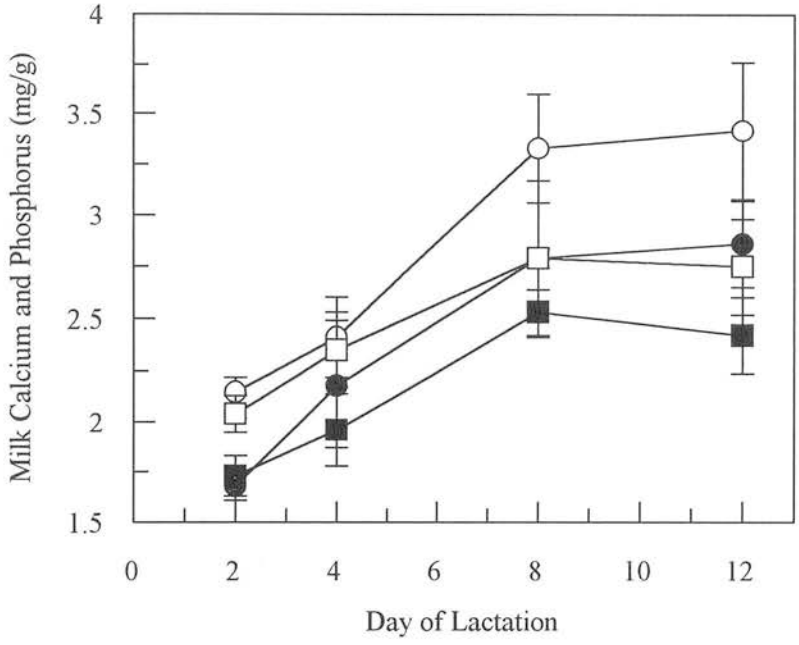

Fig. 5.8. Changes in the milk calcium (●) and phosphorus (■) contents (mg/g) of females offered diets H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

The combined calcium and phosphorus content (CaP) of FFM was significantly affected by the lactation diet and stage of lactation, increasing through lactation in group H and L to values on day 12 of 6.39 (± 0.44) and 7.74 (± 0.76) mg/g FFM respectively ($P<0.05$) (Fig. 5.10). During lactation CaP content of FFM was increased by the low protein diet ($P<0.05$) (6.65 (± 0.4) vs 5.44 (0.33) mg/g FFM). This significant difference was also evident on day 8 of lactation (7.92 (± 0.64) vs 6.49 (± 0.15) mg/g FFM).

DISCUSSION

If the effects of variations in nutrient supply on rat milk composition and lactational performance are to be investigated, it is essential that the milk sample obtained reflects that

received by the suckling young. Since prolonged milk stasis within the gland (> 4 hours) significantly reduces the milk fat content in rats (Grigor *et al.* 1986a), milk samples were obtained in this study after only a 2 hour separation of dam and litter. It was also intended that sampling during the early light phase would limit the impact of the diurnal variations in mammary lipogenesis and lactose synthesis (Williamson *et al.* 1984). All dams were milked only once because of the effect serial milking has on the milk micro-nutrient content (Keen *et al.* 1980).

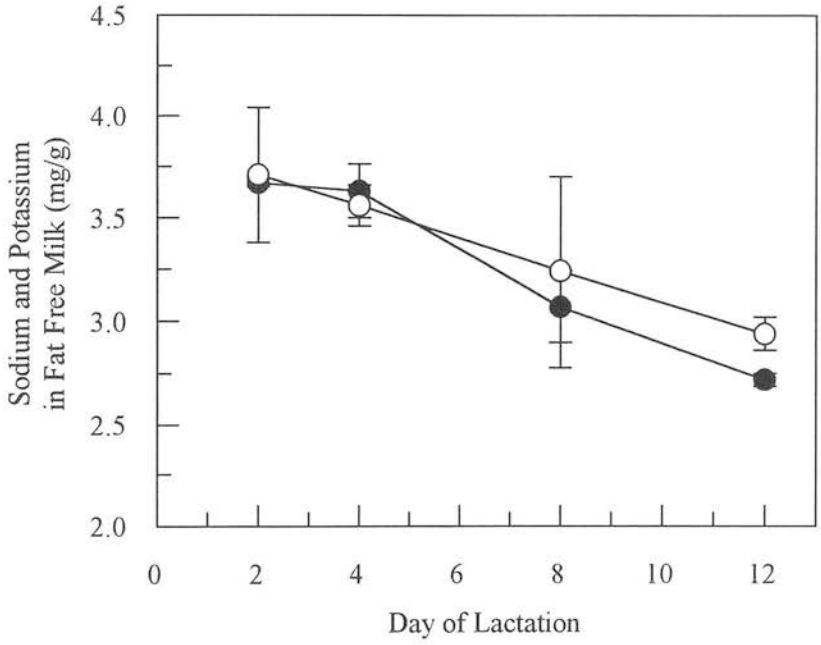

Fig. 5.9. Changes in the combined sodium and potassium contents of fat free milk (mg/g) of females offered diets H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

Furthermore, in many studies investigating milk composition, lactating rodents are often injected with exogenous oxytocin in order to obtain a milk sample, with doses of oxytocin ranging from 0.1 - 40 i.u. However, the use of oxytocin in doses greater than 1 i.u. has been suggested to significantly influence milk composition, with sodium and chloride concentrations increasing following an elevation of paracellular ion transport (Linzell *et al.*

1975). Although the dose of oxytocin used in this study (5 i.u.) exceeded that suggested by Linzell *et al.* (1975), the milk sodium:potassium ratio ranged from 1:1.8 - 1:3.7 which is considerably greater than the 1:1 reported by Chalk *et al.* (1979) and is close to the 1:3 reported for many species and intra cellular fluid (ICF) (Peaker 1977b). In this study therefore, since the combined milk sodium/potassium concentration declined during lactation on both dietary treatments and the concentration of potassium relative to sodium was higher than previously reported, the oxytocin treatment was taken to have had little impact on milk composition.

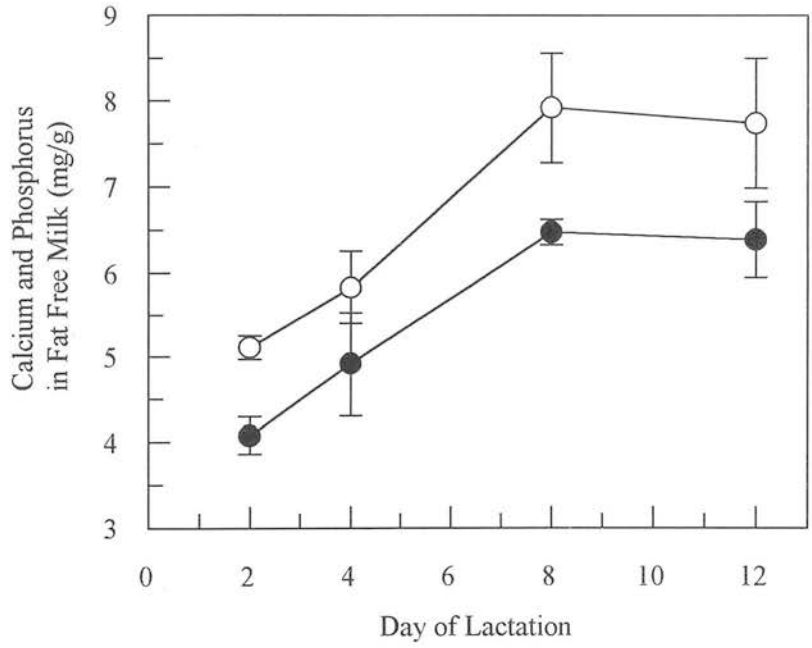

Fig. 5.10. Changes in the combined calcium and phosphorus contents of fat free milk (mg/g) of females offered diets H (●) and L (○) during lactation. Four, separate females from each dietary treatment were used to provide milk samples on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

The results of the current study are in agreement with those of earlier experiments in this thesis and confirm that severe reductions in the protein:energy ratio of diets offered to lactating rats can drastically impair feed intake and therefore lactational performance (Chapters 2 and 4). Such dietary protein restriction can suppress intake and milk secretion if

encountered both following parturition (Friggens 1990, Naismith *et al.* 1982) or during established lactation (Grigor *et al.* 1987a, Sainz *et al.* 1986a). Protein restricted females can supplement this dietary supply by mobilising their endogenous reserves of protein (Friggens 1990, Naismith *et al.* 1982, Chapters 2 and 4) and in females offered a diet of similar protein:energy ratio as used in this study (L), such protein catabolism can supply up to 1.0 g/d of endogenous protein (Chapter 4), although at such a rate the limited reserves are thought to be depleted within 6 - 9 days.

During lactation, rats will also use their adipose stores that were accumulated during gestation (Naismith *et al.* 1982, Chapter 2) to provide a supplementary supply of energy and milk fat precursors (Davies *et al.* 1983, Rolls *et al.* 1986). It has been suggested, that reductions in the dietary protein:energy ratio during lactation, combined with this endogenous energy supply, precipitates a suppression of nutrient intake, and thus lactational performance, to prevent an imbalance of protein and energy yielding nutrients which under such conditions cannot be disposed of. Such an interaction between dietary and endogenous nutrients would account for the reduced feed intake and lactational performance seen in this study with diet L. However, from the earlier studies in this thesis it has been shown that dams offered a diet of comparable protein:energy ratio as diet H increased their feed intake and lactational performance throughout lactation (Chapter 2), even though they were mobilising considerable quantities of body fat. The results for group H in this study followed a similar pattern and suggest that high rates of fat loss, *per se*, are not incompatible with a capacity to enhance feed intake during lactation.

In this study the feeding of diet H promoted an increase in mammary size. This is likely to have been allied to an increase in mammary metabolism, particularly lipogenesis (Williamson 1980) and protein synthesis (Jansen *et al.* 1986, Chapter 3), associated with milk production. Earlier studies have shown that protein restriction (quantity and/or quality) during lactation prevents such organ hypertrophy and actually results in a reduction of mammary mass and cellularity (Sampson *et al.* 1984c, Turner *et al.* 1973, Chapter 4). The

results of the current study are in agreement with these findings and under such conditions mammary blood flow (Sakanashi *et al.* 1987) and protein synthesis (Sampson *et al.* 1984c, 1986, Chapter 2) might be expected to have been reduced.

In the current study, the milk from dams offered the high protein/high energy diet during lactation exhibited a distinct increase in lactose content with stage of lactation, while milk protein and fat remained relatively unchanged. These results are in agreement with those from earlier studies by Luckey *et al.* (1954), Chalk *et al.* (1979), Keen *et al.* (1981) and Nicholas *et al.* (1991). The milk lactose content on day 12 (2.65 (± 0.01) %) was close to that reported by Grigor *et al.* (1985, 1987a, 1989) on day 14 but considerably lower than that of Luckey *et al.* (1954) and Keen *et al.* (1981). Alternatively, the milk protein contents reported here (89.4 - 91.9 mg/g) were similar to those described by Luckey *et al.* (1954), Keen *et al.* (1981) and Nicholas *et al.* (1991), although the values shown in Fig. 5.5 are 20 % greater than those reported by Sturman *et al.* (1986) for well fed females. Since milk proteins and lactose are transported together in vesicles that originate in the golgi body, milk protein concentration is largely determined by the osmotic flow of water into these vesicles and as a result in many mammalian species there exists a close relationship between milk protein and lactose concentrations (Jenness *et al.* 1987).

That milk is isosmotic with blood is now widely accepted (Peaker 1983). Since the osmotic pressure of blood is relatively constant, it is essential that the milk's osmotic pressure is maintained and this occurs through the collective activity of the milk constituents. However, this osmotic pressure is largely determined by the lactose and diffusible ion concentrations, particularly sodium and potassium, and across mammalian species there exists a strong inverse relationship between milk lactose and sodium/potassium concentrations. The osmotic passage of water into the golgi body and secretory vesicles following lactose synthesis is the major mechanism of water movement into milk (Linzell *et al.* 1971) and as such lactose synthesis determines milk volume. This flow of water establishes a potential difference (P.D.) across vesicular membranes and it is against this P.D. that the sodium/potassium

concentrations are determined (Davies *et al.* 1983, Peaker 1977a). The changes in milk lactose and sodium/potassium (mainly sodium) concentrations reported in this study are in close agreement with this inverse relationship.

In quantitative terms, fat is the most important nutrient in rat milk and in the present study the consistently high milk fat concentrations of group H (15 - 18 %) are similar to those from earlier investigations (Chalk *et al.* 1979, Grigor *et al.* 1985, 1987a, 1989, Keen *et al.* 1981). However, the values reported here are also considerably higher than those of Nicholas *et al.* (1991), who used lower dietary fat contents than in the present study, and Luckey *et al.* (1954), where excessive separation of dam and litter may have promoted a reduction in milk fat content (Grigor *et al.* 1986a).

Reductions in the dietary protein:energy ratio can significantly influence the milk fat concentration and in the current study, the feeding of the low protein/high energy diet (L) increased the milk fat content during lactation compared to diet H (205.5 (± 13.5) vs 166.8 (± 5.8) mg/g), with the fat concentrations also being significantly different on days 4 and 8. An increase in milk fat content has been previously reported for rats protein restricted throughout lactation (Crnic *et al.* 1978, Mueller *et al.* 1946, Sturman *et al.* 1986), while protein restriction during established lactation (20 vs 10% casein) (Grigor *et al.* 1985, 1987b, 1989) resulted in milk fat contents on day 14 that were comparable to those on day 12 in this study (210 vs 181 mg/g).

This increase in milk fat content by females under severe dietary protein restriction during lactation (Fig. 5.6), may simply result from a reduction in milk volume whilst milk fat synthesis continues unaltered. Alternatively, it may also be an attempt by such females to dispose of surplus energy yielding nutrients and therefore relieve the metabolic embarrassment they encounter as a result of the imbalanced supply of protein and energy yielding nutrients that are derived from dietary and endogenous sources. However, this ability may be limited because of inhibited mammary gland lipogenesis that could be promoted by the substantial supply of dietary and endogenous lipid (Grigor *et al.* 1980). The nutrient supply received by

the suckling young may therefore be maintained during periods of impaired milk production by an increase in milk fat content. In lactating sows such an increase is achieved by excessive body fat mobilisation (Noblet *et al.* 1986), while the increase in the fat content of rat milk following a reduction in litter size is not the result of altered lipid metabolism but simply a reduction in milk demand (Grigor *et al.* 1986b).

For the protein restricted females in the current study, in addition to the tendency for the milk fat content to be increased, the reduced supply of protein to the mammary gland also resulted in an immediate and significant fall in total milk protein content (12 - 15 %), which, apart from day 8, remained at this reduced level throughout lactation. These changes in milk protein content are supported by the results of earlier studies in which severe protein restriction throughout lactation lowered the protein concentration of rat milk during mid - late lactation by 27 - 30% (Crnic *et al.* 1978, Sturman *et al.* 1986), while reductions in dietary protein quality lowered milk protein content by 20% (Grimble 1981). It is likely that protein restriction exerts it's effect on milk protein through a simple reduction in amino acid supply, since protein and energy restriction was shown to reduce milk production but not protein content (Kliewer *et al.* 1987). Despite the growing body of evidence supporting the adverse effects of protein restriction on milk composition, the author is unaware of any other published data concerning the impact of severe protein restriction following parturition on changes in milk protein content throughout lactation.

In contrast to the above effects of protein restriction following parturition, reductions in dietary protein supply during established lactation, although restricting milk production, has been reported to have no significant impact on total milk protein content but reduces the whey protein concentration, particularly alpha-lactalbumin (Grigor *et al.* 1985, 1987a, 1989). In this study alpha-lactalbumin levels during lactation were not recorded, but since protein restriction reduced total protein content it might be thought that such restriction would also have reduced milk alpha-lactalbumin. The reasons for these variable effects of dietary protein restriction on milk protein content during lactation is, however, unknown.

One of the aims of this study was to investigate whether the use of maternal protein reserves during lactation, in response to severe protein restriction, was able to influence milk composition. From an earlier study, a diet of similar protein:energy ratio to that of diet L has been shown to severely deplete the protein reserves of female rats during a 12 day lactation period (Chapter 2). Since, in this study, group L lost considerable amounts of body weight, it might be expected that part of this weight loss would be associated with lean tissue. However, despite this supply of endogenous protein, especially during early lactation, milk protein content was significantly reduced by protein restriction. Therefore it can be concluded that maternal protein reserves could not prevent milk quality from being significantly impaired. Furthermore, since the feeding of diet L during lactation resulted in an impaired lactational performance (milk secretion) after day 1, it might be interpreted that the lower milk protein content during early lactation also reflected a reduced protein secretion. Although dietary protein deficiency has been shown to significantly reduce mammary protein synthesis during mid lactation (Geursen *et al.* 1987, Sampson *et al.* 1986, Chapter 3), the impact that a reduced amino acid supply has on protein synthesis during early lactation is unknown. Jansen *et al.* (1986) reported that a reduction in protein quality had no measurable effect on mammary protein synthesis during the first 6 days of lactation. Even after milk protein has been synthesised, considerable post-translational degradation of milk protein can occur prior to secretion (Hasan *et al.* 1982) and this has been suggested to be a means of controlling milk protein production following synthesis (Wilde *et al.* 1986). This degradation appears to be regulated via a negative feedback mechanism involving a milk whey protein (Wilde *et al.* 1989b). Whether, despite a supply of endogenous protein, dietary protein deficiency and possibly an imbalance in amino acid supply influences the rate of milk protein degradation and thus milk protein content is unknown. Further information on changes in milk protein degradation during severe protein under-nutrition is required.

In contrast to the above changes in milk protein and fat concentrations, there was no immediate effect of severe protein restriction following parturition on the lactose concentration

of rat milk in the current study. However, while the milk lactose content of group L followed a similar pattern to that of group H up to day 4, thereafter this pattern was severely disrupted and the milk lactose content was reduced by the continued feeding of diet L. Whilst both the precise point at which the milk lactose content was reduced by the dietary protein restriction and the associated changes in mammary gland metabolism involved are unknown, it should be noted that the sample points, day 4 and 8, were chosen because they were either side of the stage of lactation at which it was expected that maternal protein reserves would have been depleted (Chapter 4).

Although the mechanism(s) involved in the fall of milk lactose content following prolonged dietary protein restriction is unknown, one possibility involves the milk whey protein alpha-lactalbumin, an important component of the lactose synthase complex (Kuhn 1983). Since a shortage of dietary protein can reduce alpha-lactalbumin synthesis (Grigor *et al.* 1985, 1987a), such a reduction could have consequently impaired mammary gland lactose production. In support of this proposal, evidence from Grimble *et al.* (1987) suggests that the impaired lactose synthase activity in rat dams offered low quality protein diets during lactation could be improved *in vitro* through the addition of bovine alpha-lactalbumin, although at a level of 10 mg/g tissue. However, such a regulation is thought to be unlikely, except during lactogenesis, as alpha-lactalbumin concentrations in rat milk (2 - 8 mg/g) are generally in excess of the level (1 mg/g) required to fully activate the lactose synthase complex (Kuhn et al. 1980). Alternatively, lactose synthesis in the golgi body could have been impaired by a fall in the mammary gland's glucose supply (Williamson *et al.* 1984), although this again seems unlikely given the high carbohydrate content of diet L and the comparable feed intakes of day 3 and 7 (Fig. 5.1). Whether a reduction in glucose transport into the cell via the monosaccharide transport system, which has been suggested to be the rate limiting step in glucose utilisation (Threadgold *et al.* 1981), was associated with this fall in milk lactose also remains to be elucidated.

Whatever changes in mammary metabolism are involved in the dramatic fall of milk lactose content, it is interesting to note that this reduction occurred during a period when the rate of litter weight gain had fallen by over 30% (Fig. 5.2) and during which time there would have been a substantial depletion of maternal protein reserves. How a curtailed endogenous protein supply could influence mammary carbohydrate metabolism is unclear, but if the endogenous amino acids were used to any extent as gluconeogenic precursors, a reduced supply would possibly limit the activity of the lactose synthase complex.

It has already been mentioned that the osmotic pressure of milk is determined primarily by the lactose and diffusible ion concentrations, particularly sodium and potassium. Under normal conditions, following changes in one constituent the osmotic pressure is maintained by compensatory changes in the others. When continued feeding of diet L resulted in the significant fall in lactose concentration (day 4 - 8), it might be expected that the sodium/potassium concentrations would have been adjusted to maintain the milk's osmotic pressure. However, during this period their concentrations also fell in both whole and fat free milk.

Although the other ions measured in this study, calcium and phosphorus, can also contribute to the osmotic pressure, this ability is limited because the bulk of these ions (calcium 97 %, phosphorus 78 %) are associated with the non-diffusible phase (Davies *et al.* 1983). Therefore, despite the fact that their concentrations were increased considerably in both whole and fat free milk, the impact this increase could have on the osmotic pressure is determined by its partitioning between the diffusible and non-diffusible phases. Since the osmotic pressure must be maintained and because of the uncertainty regarding the partitioning of the extra calcium and phosphorus ions, it is possible that a milk constituent that contributes to the osmotic pressure and compensates for the fall in milk lactose has been ignored. Whilst the importance of the other milk components involved in the maintenance of the osmotic pressure may be increased under such conditions, such a fall in lactose production may

promote a decline in milk volume (Grimble *et al.* 1987) and thus an increased milk fat and protein concentration (Figs. 5.5, 5.6).

Predictability of Milk Yield

Although in this study, the weight gain of a standardised litter has been used as a qualitative index of milk production (lactational performance), this cannot be accurately used to predict milk yield since no consideration is given to pup maintenance requirements or possible variations in nutrient supply that result from alterations in milk composition. In recent years, equations have been developed, for use in nutritional studies concerning lactation, that estimate rat milk yield from both pup weight and pup weight gain (Grigor *et al.* 1987a, Sampson *et al.* 1984b). Although the authors suggest that such equations are equally applicable to milk yield estimation from severely undernourished dams, this assumes that the nutritional challenge does not compromise milk composition. Since in this study we have reported that severe protein restriction from parturition can quickly alter milk composition and therefore quality, the use of these equations under such conditions must be in question.

Using data from this study and the equations described by Sampson *et al.* (1984b) and Grigor *et al.* (1987a), females offered diet H during lactation and milk sampled on day 8 had estimated milk yields over the preceding 24 hours (day 7 - 8) of 34.4 and 39.5 g/d respectively. This slight discrepancy in estimated milk yield (12 %) is largely the result of a lower estimate of pup maintenance requirement used by Sampson *et al.* (1984b).

Alternatively, because of possible variations in nutrient supply, using data for milk composition from the same females, it is also possible to estimate the milk yield needed to supply the pup maintenance and gain requirements over 24 hours. In such an estimation of milk yield from milk composition and litter protein requirements, the litter protein need for maintenance was determined using the equation derived from Emmans *et al.* (1988) and Friggens (1990) ($10 \times 0.07^{-0.27} \times$ Body Protein), a digestibility of milk protein of 0.95 (Radcliffe *et al.* 1978) and an efficiency of absorbed protein use of 0.85 (McDonald *et al.*

1981). Litter protein gain on day 7 (day 7 - 8) was estimated by interpolation from the litter protein mass on day 4 (-5.6 + 0.176 Litter Weight; r^2 = 95.9 %, $P<0.05$) and the protein mass on day 8 (25.84 (± 1.1 g) (Fig. 5.3). The protein yield required to satisfy such growth and maintenance requirements was then converted to milk volume using the measured milk protein concentration.

Using the above calculations, the milk yield on day 7 was estimated to be 43.7 g/d, which was 10 and 22% higher than the calculated yields using the equations of Grigor *et al.* (1987a) and Sampson *et al.* (1984b) respectively.

When these equations are applied to data from females offered diet L and used for milk sampling on day 4, milk yields for the previous 24 hours (day 3 - 4) were estimated to be 17.6 and 15.5 g/d respectively, and as might be expected were considerably lower than the 23.9 and 20.9 g/d calculated for group H.

The milk yield for group L on day 3 can also be calculated using the litter protein requirements and calculations previously described for group H. In this calculation for group L, Litter Protein on Day 2 was estimated from the equation -5.0 + 0.165 Litter Weight (r^2= 90.0 %; $P<0.05$) and the litter protein mass on day 4 of 12.19 (± 0.50)g. Using these calculations and assumptions, the milk volume required to supply sufficient milk protein was 30.5 g/d, which was 42 and 49 % greater than the yields calculated using the equations from Grigor *et al.* (1987a) and Sampson *et al.* (1984b) respectively. Although this required milk volume may be slightly overestimated, unlike group H it can be clearly seen that since milk quality, particularly milk protein, was significantly reduced by dietary protein restriction, estimations of milk yield simply from litter weight and weight gain dramatically underestimate the volume of milk required to supply sufficient nutrients to support litter maintenance and weight gain. It can therefore be concluded that although such equations are useful for the estimation of milk yield from well nourished dams, when dietary conditions results in an altered milk composition the application of such methods is questionable.

The estimated milk yield for group L of 30.4 g/d, represents a litter milk protein requirement of 2.3 g/d. Although there may some degree of over estimation in this calculated requirement, it is interesting to note that in the same females this is actually greater than their dietary protein intake (1.96 (± 0.17) g/d). These estimations suggest that such females require an additional supply of protein to allow lactation to proceed successfully. This additional supply of protein will be derived from maternal protein reserves and it has been previously reported that during early lactation such protein restricted females can supply up to 1.0 g/d of endogenous protein (Chapter 4).

In summary it can be concluded that severe dietary protein restriction during lactation not only impairs milk production through a suppression of feed intake and a limiting protein supply, it also significantly adjusts milk composition. Protein restriction following parturition considerably reduces milk protein concentration while increasing milk fat content. Milk lactose content is not initially affected, but continued protein restriction results in a dramatic fall in milk lactose content, while at the same time concentrations of fat and protein rise. The reasons behind such alterations in milk lactose content and thus mammary gland carbohydrate metabolism are unknown, but it is thought that the exhaustion of maternal protein reserves and therefore the supply of endogenous protein to the gland is involved. Recently developed methods to estimate milk production by lactating rats from litter weight gain and litter weight can be used to provide useful information on the yield of well nourished dams. However under conditions in which both milk volume and composition may be adjusted, their application is in doubt.

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CHAPTER SIX

EXPERIMENT E4

THE EFFECT of DIETARY PROTEIN RESTRICTION DURING LACTATION on TISSUE PROTEIN SYNTHESIS in RATS and the CHANGES in MUSCLE PROTEIN TURNOVER INVOLVED in the MOBILISATION of MATERNAL PROTEIN.

The Effect of Dietary Protein Restriction During Lactation on Tissue Protein Synthesis in Rats and the Changes in Muscle Protein Turnover Involved in the Mobilisation of Maternal Protein.

ABSTRACT

This study was undertaken to investigate the changes in muscle protein turnover involved in the rapid mobilisation of protein in rats subjected to severe protein restriction during lactation. Estimates of mammary gland and liver protein synthesis were also made during lactation. Multiparous female Sprague-Dawley rats, caged individually following mating, were offered a high protein diet (H; 215 gCP/kg DM) ad libitum until parturition. Following parturition, half the females continued to receive diet H, whilst the remainder were offered a diet low in protein (L; 90 gCP/kg DM) ad libitum. On days 2, 4 , 8 and 12 of lactation groups of females were used in the estimation of tissue protein synthesis (flooding dose of [^{3}H] phenylalanine) immediately after a milk sample had been obtained. Rates of muscle protein synthesis were unchanged during lactation in group H. The feeding of diet L during lactation reduced the muscle protein synthesis on day 12 to rates that were lower than group H and also the rate on day 2 ($P<0.01$). However, this fall in muscle protein synthesis was not rapid and muscle FSR was different to group H only from day 8 ($P<0.05$). Estimated rates of mammary protein synthesis appeared to be generally unchanged by dietary treatment or stage of lactation. Liver FSR was also unchanged by dietary protein supply or stage of lactation. The effect of dietary protein restriction on liver size and protein content during lactation influenced liver ASR, and on days 8 and 12 of lactation liver ASR was lower in group L than in group H ($P<0.001$). The loss of muscle protein in rats fed diet L during lactation (133 mg) occurred mainly between day 2 and 8 of lactation. This rapid loss of muscle protein during early lactation was primarily associated with a dramatic increase in degradation (13.0 %/d), with the decline in synthesis having a much

smaller role. A decline in muscle protein degradation during the latter half of lactation was part of the mechanism that prevented excessive muscle protein catabolism. It is thought that the estimation of mammary protein synthesis in this study was impaired by the milk sampling procedure previously used.

INTRODUCTION

It is now well recognised that lactating females can supplement their available nutrient supply by mobilising endogenous reserves of protein. The use of such reserves by dairy ruminants is most frequently associated with early lactation, when the gap between feed intake and milk yield creates an imbalance between nutrient supply and demand (Belyea *et al.* 1978), while in rodents such maternal protein loss is promoted by periods of protein undernutrition (Naismith *et al.* 1982, Chapter 2) during both gestation and lactation. Since skeletal muscles are the primary site of protein mobilisation (Swick *et al.* 1977), several studies, involving both ruminants and non-ruminants, have investigated the possible adaptive mechanisms of muscle protein turnover involved during lactation.

In lactating ruminants, the net loss of hind limb muscle protein has been reported to be associated with a fall in protein synthesis in goats (Baracos *et al.* 1991,Champredon *et al.* 1990) and in sheep with an increase in degradation (Vincent *et al.* 1985). Other workers using lactating sheep have concluded that the mechanism involved depended on the individual muscle concerned (Bryant *et al.* 1982). Similar studies involving lactating rodents could not attribute the protein loss from protein restricted females to either alterations in carcass protein turnover (Sainz *et al.* 1986b) or muscle protein synthesis (Sampson *et al.* 1984c), although from measurements of urinary 3-methylhistidine excretion, Sainz *et al.* (1984) suggested that an increase in protein degradation was involved. As a result of these studies, the possible controlling mechanisms responsible for muscle loss remain uncertain and confused.

From an earlier study in this thesis (Chapter 3) it was suggested that the loss of muscle protein from female rats subjected to severe protein restriction during the first 12 days

of lactation was associated with both a fall in synthesis and an increase in degradation, with the increase in degradation being quantitatively more important. In that study, muscle protein synthesis was estimated at the start and end of the 12 day period of lactation, while the rate of protein degradation was calculated assuming a constant rate of muscle protein loss. However, from recorded changes in lactational performance (litter weight gain) (Chapters 2 and 4) and the suggestion that in similarly treated females the loss of muscle protein occurs rapidly during early lactation (1.0 g/d) before reaching an apparent metabolic limit (days 6 - 9) after which such protein loss is severely impaired (Chapter 4), this assumed constant rate of maternal protein loss is clearly an over-simplification of true events. It is therefore apparent that the measurement of muscle protein metabolism at two stages during lactation cannot accurately describe the changes in muscle protein turnover involved in such a pattern of mobilisation. Changes in muscle protein metabolism throughout lactation require description and will be reported in this study.

In both ruminants and non-ruminants, whole body protein turnover is considerably increased during lactation through an elevation of mammary gland, liver and gastrointestinal tract protein synthesis (Champredon *et al.* 1990, Millican *et al.* 1987). This increase is partly the result of organ hypertrophy/hyperplasia which is in turn promoted by an enhanced feed intake (Vernon 1988, Williamson 1980). In rodents, liver fractional protein synthesis (FSR; %/d) during lactation is relatively unaffected by dietary protein quantity or quality, although such liver hypertrophy, and thus absolute synthesis rate (ASR; mg/d), is prevented by dietary protein restriction (Sampson *et al.* 1984c, Chapter 3). Mammary protein synthesis (FSR and ASR) is more sensitive, and is significantly impaired by dietary protein restriction during lactation (Sampson *et al.* 1984c, Sampson *et al.* 1986), although prior restriction during gestation has been shown not to prevent protein synthesis from increasing during lactation when adequate nutrition is provided (Chapter 3).

Mammary protein synthesis, despite being crucial to both the secretion of milk protein and the maintenance of mammary integrity, has been shown to be correlated with milk

secretion (Sampson *et al.* 1985) and also increases from early to peak lactation (Jansen *et al.* 1986, Millican *et al.* 1987). However, the pattern of this increase and possible changes imposed by dietary protein restriction remain to be elucidated. Whether the suggested fall in milk secretion that results from the depletion of maternal protein reserves is also reflected in alterations in mammary protein synthesis is unknown.

The objectives of the current study were to investigate the changes in muscle protein turnover involved in the rapid mobilisation of protein from female rats subjected to severe protein undernutrition during early lactation. In addition, changes in mammary and liver protein synthesis throughout lactation will be reported.

MATERIALS and METHODS

The females used in this study were also involved in the investigation of changes in milk composition during lactation, the results for which have been reported previously (Chapter 5). A similar experimental protocol was therefore followed in both studies, with milk composition and rates of protein synthesis being estimated in the same female offered a diet of either a high (H; 215 g CP/kg DM) or low (L; 90 g CP/kg DM) protein:energy ratio during lactation.

Experimental Protocol

For a full description of the rat strain, female numbers, diet composition, feeding procedure, data collection and litter size used in this study the reader is referred to Chapter 5. Groups of females (n=4) were used on days 2, 4, 8, and 12 of lactation for the estimation of tissue protein synthesis immediately after a milk sample had been obtained. Briefly, the experimental design involved 32 multiparous female rats being offered the high protein diet H *ad libitum* from conception until parturition after which, half continued to receive diet H *ad libitum* during lactation, whilst the remainder were offered the low protein diet L. During lactation, dams on both dietary treatments were then used in the analysis of milk composition

and tissue protein metabolism. Throughout the experiment, maternal body weight and feed intake were recorded daily, whilst litter weights were recorded during the 12 day lactation period.

The milking procedure involved an initial 2 hour separation of dam and litter at the start of the light period, after which the dams were lightly anaesthetised (diethyl ether) and injected subcutaneously with 5 i.u. of oxytocin. Milk samples were then obtained from the left thoracic and abdominal teats. While the dam was still anaesthetised, *in vivo* rates of tissue protein synthesis were estimated.

Measurement of Tissue Protein Synthesis

Rates of *in vivo* total protein synthesis were measured during lactation in the gastrocnemius muscle, mammary gland and liver using the flooding dose technique of Garlick *et al.* (1980). Technical aspects of this procedure have been previously described elsewhere (Chapter 5, Appendix 1).

Fractional synthesis rates (FSR) are calculated using the equation

$$FSR\ (\%/d) = \frac{S_B \times 100}{S_A \times t}$$

where S_B and S_A are the specific activity of protein bound and free phenylalanine respectively, and t is the time elapsed between injection and tissue cooling. Absolute synthesis rates (ASR; mg/d) are calculated using the FSR, organ weight and tissue protein content.

In this study, samples of the right hand abdominal mammary gland were used in the estimation of protein synthesis since the left hand gland had received physical manipulation during the milking procedure. Once the tissue samples had been cooled in liquid nitrogen, the remaining liver and right hand mammary gland were dissected and weighed.

Tissue protein content was measured using the method of Lowry *et al.* (1951) with bovine serum albumin as a standard and the RNA concentration was estimated as described by Munro *et al.* (1969), with muscle RNA calculated using the equation of Ashford *et al.* (1986). Diethyl ether was chosen for the anaesthetic because it has been previously reported not to effect rates of tissue protein synthesis during lactation (Sampson *et al.* 1984d).

Statistical Analysis

Dietary effects on rates of protein synthesis and tissue composition were analysed by two-way analysis of variance, and by calculation of least significant differences, T tests were used to compare sample means between diets and individual days. Changes in muscle protein content during lactation were also analysed by analysis of variance, with day 1 lactation body weight as a covariate (Genstat5).

RESULTS

The effects of the lactational dietary treatments on maternal body weight loss, feed intake and lactational performance (litter weight gain) have been previously reported (Chapter 5) and are shown in Figs. 6.1, 6.2 & 6.3.

The feeding of diet L during lactation resulted in a considerably greater weight loss ($P<0.001$) by group L (Fig. 6.1). In addition, diet L resulted in a significant suppression of feed intake throughout lactation (Fig. 6.2) and, as a consequence of this reduced intake and the low dietary protein:energy ratio, litter weight gain was also significantly impaired (Fig. 6.3).

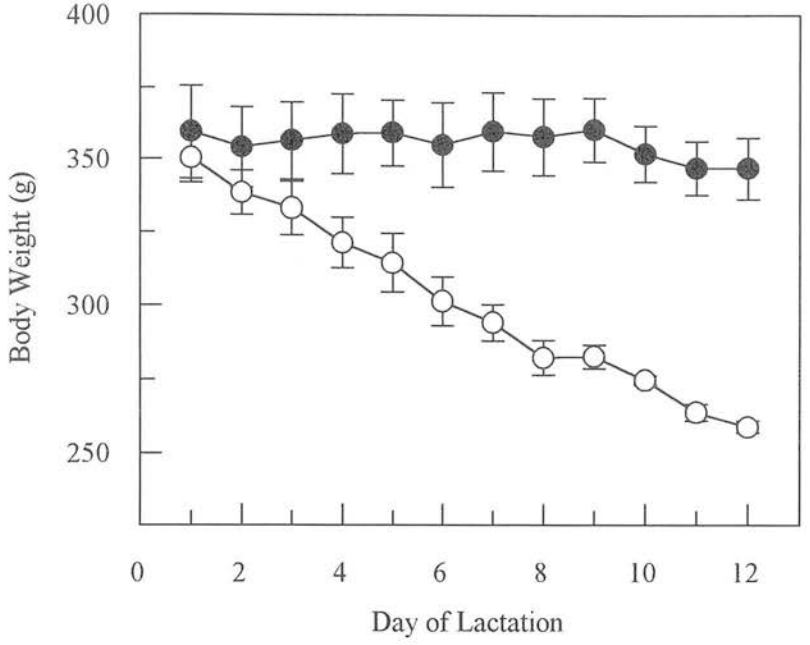

Fig. 6.1. The change in body weight between day 1 and 12 of lactation for females offered diets H (●) (n=4) and L (○) (n=4) during lactation. Females were weighed at the same time each day and each point represents a mean with SEM.

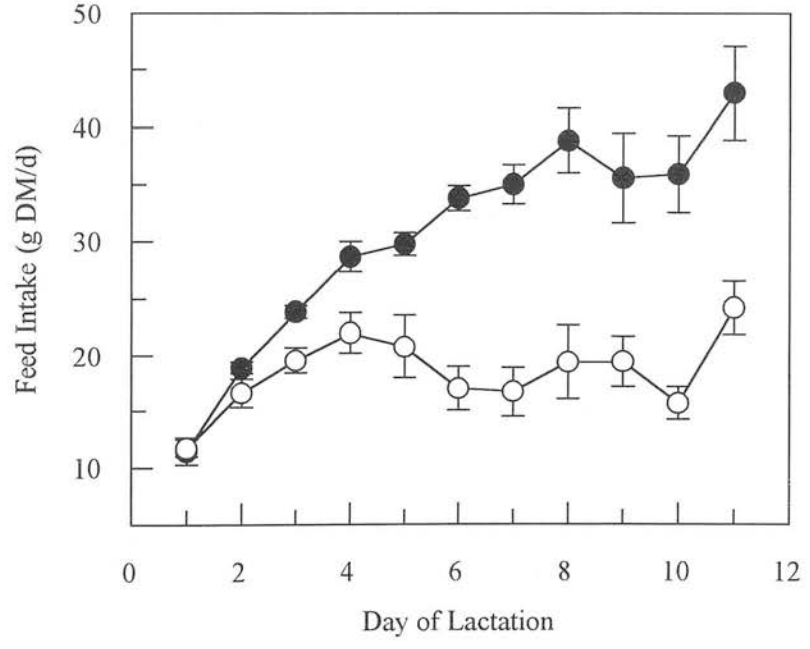

Fig. 6.2. Daily feed intake (g DM) of females offered diets H (●) and L (○) during lactation. Data for all females offered diet H and L at each stage of lactation are included and each point represents a mean with SEM.

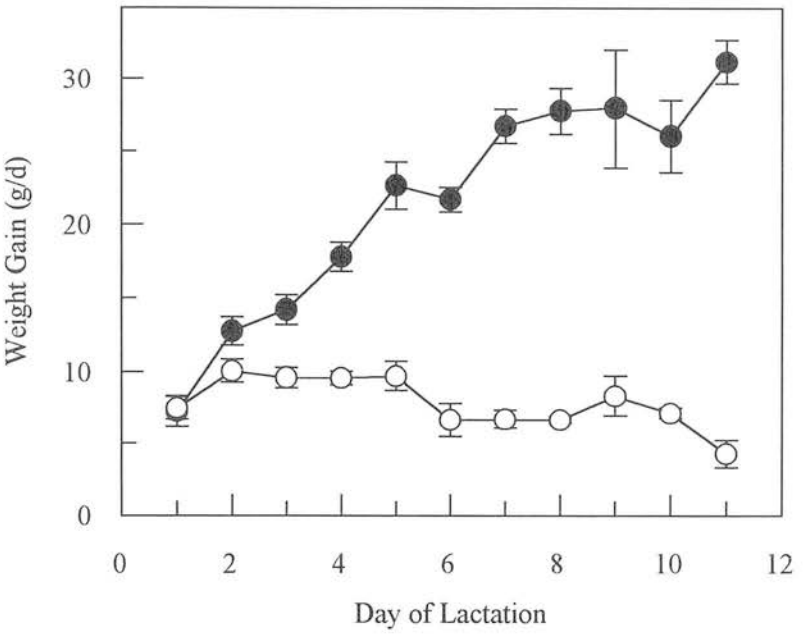

Fig. 6.3. Daily litter weight gain (g) for females offered diets H (●) and L (○) during lactation. Data for all females offered diets H and L at each stage of lactation are included and each point represents a mean with SEM.

The Effects of the Lactation Dietary Treatments on Muscle Weight, Composition and Rates of In Vivo Protein Synthesis

The weight, composition and estimated rates of protein synthesis in the gastrocnemius muscle from female rats offered diets H and L during lactation are shown in Table 6.1.

The feeding of diet H during lactation, promoted no significant change in muscle weight, protein content, RNA content or FSR and ASR in relation to stage of lactation. However, dietary protein restriction during lactation did result in significant alterations in muscle protein metabolism

Table 6.1. Effect of lactation dietary treatment on muscle weight, composition and in vivo rates of protein synthesis during lactation (Mean with SEM).

TREATMENT GROUP		H	L
Weight (g) :	Day 2	1.46 ± 0.10[a]	1.48 ± 0.10[a]
	4	1.50 ± 0.05[a]	1.27 ± 0.08[b*]
	8	1.42 ± 0.03[a]	1.17 ± 0.02[b*]
	12	1.42 ± 0.03[a]	1.13 ± 0.03[b**]
Protein (mg) :	Day 2	387.2 ± 34.9[a]	385.4 ± 22.1[a]
	4	353.9 ± 15.6[a]	317.1 ± 24.5[a]
	8	345.8 ± 25.4[a]	265.3 ± 12.9[b*]
	12	373.7 ± 29.9[a]	252.1 ± 9.5[b**]
RNA (mg) :	Day 2	1.71 ± 0.11[a]	1.55 ± 0.19[a]
	4	1.68 ± 0.16[a]	1.50 ± 0.15[ab]
	8	1.88 ± 0.02[a]	1.45 ± 0.05[ab*]
	12	1.80 ± 0.11[a]	1.21 ± 0.04[b**]
FSR (%/d) :	Day 2	2.9 ± 0.3[a]	3.3 ± 0.4[a]
	4	3.5 ± 0.2[a]	2.8 ± 0.4[a]
	8	3.6 ± 0.3[a]	2.7 ± 0.1[ab*]
	12	3.5 ± 0.2[a]	2.0 ± 0.2[b**]
ASR (mg/d) :	Day 2	11.3 ± 2.1[a]	12.8 ± 2.0[a]
	4	12.6 ± 1.3[a]	8.9 ± 1.2[ab]
	8	12.2 ± 0.7[a]	7.3 ± 0.4[b*]
	12	12.3 ± 1.9[a]	5.0 ± 0.2[b**]
RNA Activity[1] :	Day 2	6.60 ± 1.04[a]	7.59 ± 0.85[a]
	4	7.61 ± 0.84[a]	6.36 ± 1.33[ab]
	8	6.46 ± 0.32[a]	5.06 ± 0.42[b]
	12	6.84 ± 0.98[a]	4.23 ± 0.31[b*]

[1] RNA activity: mg protein synthesised/mg RNA
Means in the same row differ significantly *** P<0.001, ** P<0.01, * P<0.05
[a,b] Means in same column and block with different superscripts differ significantly P<0.05

The feeding of diet L had reduced, by day 12 of lactation, the muscle weight, protein and RNA contents of group L compared to both that of group H (P<0.01) on day 12 and to the respective values for group L on day 2 (P<0.05). The difference in muscle weight between dietary groups was apparent by day 4 (P<0.05), while muscle protein and RNA contents were significantly different by day 8. Group L muscle protein loss between day 2 and 12 of lactation was approximately 133 mg, although the bulk of this loss (120 mg) had occurred by day 8. The calculated changes, from covariate analysis, in the muscle protein

content of groups H and L during lactation confirmed the significant ($P<0.01$) reduction in muscle protein mass with dietary protein restriction and are shown in Fig. 6.4.

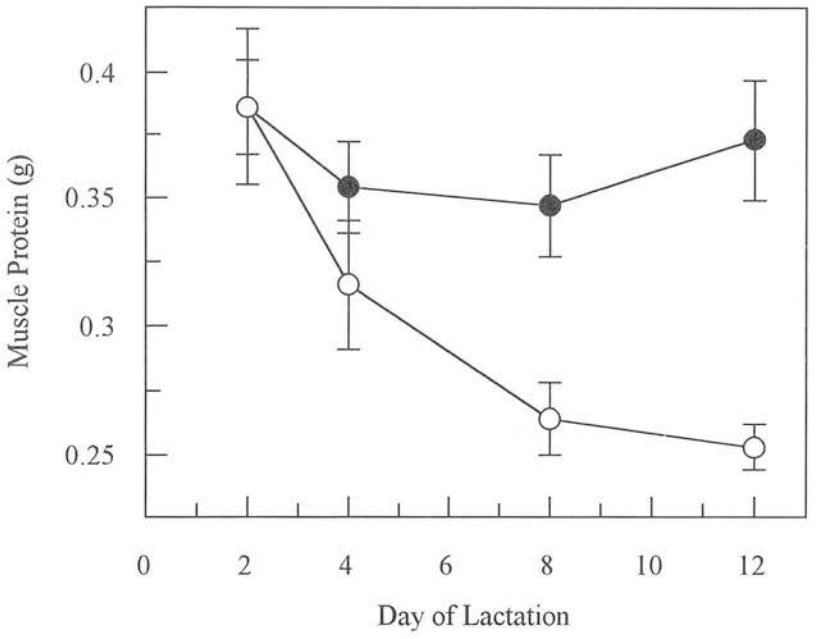

Fig.6.4. Changes in the protein content (g) of skeletal muscle (gastrocnemius), adjusted for day 1 lactation body weight, of females offered diets H (●) and L (○) during lactation. Muscles were dissected from females (n=4) that were slaughtered on either day 2, 4, 8 or 12 of lactation and each point represents a mean with SEM.

Rates of muscle protein synthesis were significantly reduced during lactation by the feeding of diet L, with the FSR and ASR of group L on day 12, being lower than both that of group H ($P<0.01$) and of group L day 2 values ($P<0.01$). However, this dietary protein restriction did not promote a rapid fall in protein synthesis and the reduction in FSR only became significant during the last 4 days (Table 6.1). This reduction in muscle protein synthesis was reflected in a decline in the RNA activity of group L, which on day 12 of lactation was lower than both that of group H ($P<0.05$) and of the muscle RNA activity of group L on day 2 ($P<0.01$) (Table 6.1).

The Effect of the Lactational Dietary Treatments on Mammary Gland Weight, Composition and In Vivo Rates of Protein Synthesis

The weight of the right hand mammary gland, it's composition and estimated rates of protein synthesis from female rats offered diets H and L during lactation are shown in Table 6.2.

Table 6.2. Effect of lactational dietary treatment on mammary gland weight[1], composition and rates of protein synthesis during lactation (Mean with SEM).

TREATMENT GROUP		H		L	
Weight[1] (g) :	Day 2	9.03 ± 0.87[a]		10.70 ± 1.10[a]	
	4	10.17 ± 1.54[ab]		8.57 ± 1.36[ab]	
	8	12.28 ± 0.96[bc]		8.28 ± 0.58[ab]*	
	12	13.63 ± 1.27[c]		7.36 ± 0.27[b]**	
Protein (g) :	Day 2	0.89 ± 0.08[a]		1.12 ± 0.16[a]	
	4	1.09 ± 0.18[ab]		0.92 ± 0.15[ab]	
	8	1.33 ± 0.14[b]		0.75 ± 0.06[b]***	
	12	1.39 ± 0.06[b]		0.69 ± 0.03[b]***	
RNA (mg) :	Day 2	53.7 ± 3.1[a]		61.4 ± 14.2[a]	
	4	71.7 ± 7.2[a]		55.7 ± 8.4[a]	
	8	112.1 ± 11.3[b]		67.6 ± 10.7[a]**	
	12	126.6 ± 6.6[b]		60.1 ± 5.5[a]**	
FSR (%/d) :	Day 2	62.4 ± 3.7[a]		51.5 ± 4.2[a]	
	4	48.2 ± 9.1[ab]		56.6 ± 9.5[a]	
	8	33.9 ± 9.3[b]		47.4 ± 2.3[a]	
	12	55.6 ± 1.7[a]		41.8 ± 3.7[a]	
ASR (mg/d) :	Day 2	566.9 ± 77.9[a]		587.9 ± 116.6[a]	
	4	521.5 ± 155.8[a]		511.7 ± 94.7[a]	
	8	485.7 ± 190.9[a]		355.0 ± 30.4[a]	
	12	775.8 ± 45.3[a]		290.3 ± 29.3[a]*	
RNA Activity[2] :	Day 2	10.48 ± 1.11[a]		10.08 ± 1.44[a]	
	4	7.25 ± 1.79[ab]		9.18 ± 1.26[ab]	
	8	4.14 ± 1.24[b]		5.96 ± 1.61[bc]	
	12	6.15 ± 0.38[b]		5.05 ± 0.80[c]	

[1] Right hand abdominal and thoracic mammary gland
[2] RNA activity: mg protein synthesises/mg RNA
Means in the same row differ significantly *** P<0.001, ** P<0.01, * P<0.05
[a,b,c]Means in the same column and block with different superscripts differ significantly P<0.05

The feeding of diet H during lactation promoted a significant increase in mammary gland weight, protein and RNA contents, whilst diet L resulted in significant mammary regression. As a consequence, on days 8 and 12 of lactation mammary gland weight ($P<0.05$), protein content ($P<0.01$) and RNA content ($P<0.01$) were greater in dams offered diet H. For both dietary groups, the mammary gland weight and protein content on day 12 were significantly different from their respective values during early lactation.

However, in both dietary groups rates of mammary protein synthesis were generally unaltered throughout lactation and, apart from day 12 ASR, were unaffected by the dietary protein:energy ratio. Although rates of protein synthesis were unchanged, estimated mammary RNA activity was significantly reduced during lactation in both dietary groups, and by day 12 had declined ($P<0.05$) to 6.15 and 5.05 mg protein/mg RNA in groups H and L respectively. For groups H and L, RNA activities were not different throughout lactation.

The Effect of the Lactation Dietary Treatments on Liver Weight, Composition and In Vivo Rates of Protein Synthesis

The liver weight, composition and rates of protein synthesis from female rats offered diets H and L during lactation are shown in Table 6.3.

The feeding of diets H and L during lactation had similar qualitative affects on liver size and composition as those previously described for the mammary gland. During lactation, the feeding of diet H promoted considerable liver anabolism such that by day 12, liver weight ($P<0.01$), liver protein ($P<0.001$) and RNA contents ($P<0.01$) had all increased compared to that on day 2. On the other hand, the feeding of diet L resulted in a reduction in liver size during lactation, such that by day 8 liver weight ($P<0.001$), liver protein and RNA contents ($P<0.01$) of group L were all lower than those of group H.

Table 6.3. Effect of lactational dietary treatment on liver weight, composition and rates of protein synthesis during lactation (Mean with SEM).

TREATMENT GROUP		H	L
Weight (g) :	Day 2	11.68 ± 0.76[a]	12.20 ± 0.42[a]
	4	12.06 ± 1.01[a]	11.22 ± 0.51[ab]
	8	14.49 ± 0.59[b]	10.01 ± 0.24[b***]
	12	17.20 ± 0.73[b]	10.03 ± 0.61[b***]
Protein (g) :	Day 2	2.28 ± 0.17[a]	2.11 ± 0.20[a]
	4	2.23 ± 0.24[a]	1.85 ± 0.06[ab]
	8	2.82 ± 0.06[b]	1.83 ± 0.07[ab**]
	12	3.21 ± 0.19[b]	1.63 ± 0.05[b***]
RNA (mg) :	Day 2	127.2 ± 10.4[a]	125.2 ± 2.6[a]
	4	121.4 ± 3.4[a]	113.2 ± 4.7[ab]
	8	135.3 ± 3.4[a]	102.0 ± 1.7[bc**]
	12	155.6 ± 6.5[b]	98.2 ± 2.8[c**]
FSR (%/d) :	Day 2	105.6 ± 7.6[a]	84.5 ± 6.8[a*]
	4	79.0 ± 5.5[b]	82.0 ± 8.4[a]
	8	84.9 ± 3.4[b]	82.6 ± 6.6[a]
	12	79.6 ± 4.1[b]	81.4 ± 8.3[a]
ASR (g/d) :	Day 2	2.4 ± 0.2[a]	1.8 ± 0.2[a*]
	4	1.8 ± 0.2[b]	1.5 ± 0.1[a]
	8	2.4 ± 0.1[a]	1.5 ± 0.1[a***]
	12	2.5 ± 0.1[a]	1.3 ± 0.2[a***]
RNA Activity[1] :	Day 2	19.07 ± 1.87[a]	14.21 ± 1.68[a*]
	4	14.42 ± 1.30[b]	14.94 ± 1.46[a]
	8	17.83 ± 1.33[ab]	14.88 ± 1.51[a]
	12	16.40 ± 0.89[ab]	13.59 ± 1.55[a]

[1] RNA activity: mg protein synthesised/mg RNA
Means in the same row differ significantly *** P<0.001, ** P<0.01, * P<0.05
[a,b,c] Means in the same column and block with different superscripts differ significantly P<0.05

Liver FSR (%/d), apart from that on day 2 in group H, was unaffected by the dietary protein:energy ratio, and in both dietary groups remained relatively unaltered throughout lactation. In group H, the higher liver FSR recorded on day 2 of lactation, resulted in a significantly greater liver ASR (mg/d) than that measured on day 4 and also the rate on day 2 for group L. However, associated with the significant increase in liver weight and protein content from day 4 of lactation in group H (Table 6.3), liver ASR was also significantly higher on days 8 and 12. Consequently, during this period the liver ASR of group H was also higher than that of group L (P<0.001), which was not significantly changed

during lactation. The activity of liver RNA, apart from that on day 2 in group H, was unaffected by the dietary protein:energy ratio and remained unchanged throughout lactation in both dietary groups.

DISCUSSION

Methodology

The female rats involved in the current study were also used to investigate the effects of dietary protein restriction on the composition of rat milk during lactation (Chapter 5). The *in vivo* rates of protein synthesis were estimated immediately after each female had been used to provide a milk sample. Despite the plethora of studies investigating changes in milk composition and rates of tissue protein synthesis during lactation in rodents, the author is unaware of any studies in which these two investigations have been combined into one experiment involving the same female. The impact of the sampling procedure, particularly the use of exogenous oxytocin, on the subsequent estimation of protein synthesis, particularly in the mammary gland, was therefore unknown at the start of this study.

When a diet of comparable protein:energy ratio as diet H was offered to female rats during an earlier experiment, rates of mammary FSR were increased from 59 - 92 %/d between day 1 and 13 of lactation, although the time-course of this increase was not studied (Chapter 3). This increase confirmed the elevation in FSR reported by Jansen *et al.* (1986) between day 1 and 12 of lactation, and the day 13 FSR was comparable with previously reported peak rates in well fed females of 92 %/d (Jansen *et al.* 1986), 110 %/d (Sampson *et al.* 1984c) and 83 %/d (Sampson *et al.* 1986). This increased FSR would also be associated with increased mammary ASR when combined with the gain in mammary weight during lactation (Jansen *et al.* 1986).

In the current study however, despite estimated rates of mammary FSR in both dietary groups on day 2 of lactation (52 - 62 %/d) being similar to those previously reported during early lactation (Jansen *et al.* 1986, Chapter 3), in group H the expected increase in

mammary protein synthesis between day 2 and 12 of lactation was absent and at one stage mammary FSR actually exhibited a substantial and significant fall. Mammary FSR in group L was also unchanged during lactation and rates of mammary ASR in both groups reflected the situation seen with FSR. These observations suggest that the estimates of mammary protein synthesis reported here were influenced by the procedure used to sample milk. Rates of protein synthesis in the liver and muscle were however comparable with those reported in earlier studies from both this (Chapter 3) and other laboratories (Jansen *et al.* 1986, Sampson *et al.* 1984c) and there is therefore no reason to think that these estimates had been compromised by the milking regime adopted.

A further indication of the likelihood that there were errors associated with the estimated rates of mammary protein synthesis reported in this study comes from a consideration of the apparent yields of milk and milk protein. Using the equation developed by Sampson *et al.* (1984c) and the milk protein content (91.1 mg/g; Chapter 5) of females offered diet H and slaughtered on day 8 of lactation, milk and milk protein yields over the preceding 24 hours were calculated to be 34.4 and 3.13 g/d respectively. However, this compares with a total mammary protein synthesis of 0.97 g/d (2 x mammary ASR, Table 6.2). Even ignoring the proportion of endogenous protein synthesised and the possible degradation of milk protein prior to secretion (Hasan *et al.* 1980), there is a considerable difference between milk protein yield and estimated mammary production. Although other workers have reported a similar but smaller disparity between calculated and estimated mammary protein output (Sampson *et al.* 1986), this difference has been exaggerated by the inaccurate rates of mammary protein synthesis reported in this study.

Although the milking procedure therefore seems to have had adverse effect on the accurate estimation of *in vivo* mammary protein synthesis, the procedure was initially designed to limit, as far as possible, such an affect. The time of day at which a milk sample was obtained was not thought to be crucial since, unlike lactose and lipid synthesis (Williamson *et al.* 1984), milk protein synthesis does not exhibit diurnal variations (Sampson

et al. 1984c), while the period of dam and litter separation was limited to prevent an effect of milk stasis (Grigor *et al.* 1986a). In addition, the collection of milk via physical manipulation of the gland and the estimation of mammary protein synthesis were applied to different sides whilst diethyl ether has been shown not to influence rates of tissue protein synthesis (Sampson *et al.* 1984d). Given these precautions, it seems most likely that for the adverse effects on estimated mammary protein synthesis, the use of exogenous oxytocin appears to have been primarily responsible.

The mechanism by which a large dose of exogenous oxytocin might impair protein synthesis in the mammary epithelial cell is at present unknown. However, the lower protein bound (S_B) specific activity recorded in this study, suggests that the problem is associated with a reduction in [^{3}H] phenylalanine incorporation into protein and not with levels of the label in the tissue free pool. Such an inhibition may have been expressed at the translocation stage of protein synthesis because, although during lactation mammary RNA content (mg/g) was increased to levels previously reported (Sampson *et al.* 1986, Chapter 3) the activity of this RNA was significantly reduced (Table 6.2). Although the factors involved in impairing mammary protein synthesis during artificial milking are unknown, the procedure possibly creates a cellular environment in which essential ingredients (e.g. ATP, amino acids, co-factors) for peptide bond formation are limiting. Further work into the possible mechanism(s) involved is required.

Although the flooding dose technique has been used successfully in this and other laboratories for studying mammary protein synthesis, the results of the current study cannot be used as an accurate representation of changes in mammary protein synthesis during lactation, although the observations in other tissues do not seem to have been compromised.

Lactational Performance and Hepatic Protein Synthesis

In the current study, dams offered the high protein/high energy diet during lactation increased their feed intake throughout, and by day 12 intake was approximately 43 g DM/d.

This elevated nutrient supply, in addition to supporting increasing litter growth throughout lactation (Fig. 6.3), promoted considerable hypertrophy of the mammary gland and liver. However such an increase in feed intake during lactation is thought to have been limited by the lower protein:energy ratio of diet L (Fig 6.2) and, as well as impairing lactational performance, prevented such organ hypertrophy (Tables 6.2 & 6.3). In fact, the dietary protein restriction promoted considerable tissue regression during lactation and supports the findings of earlier studies involving lactating rats (Sampson *et al.* 1984c, Chapter 2). The mammary gland regression is possibly associated with both a decline in mammary mass (Turner *et al.* 1973) and cellularity (Chapter 4).

The results of the current study, support the suggestion that in lactating rodents liver protein synthesis (%/d) is not influenced by dietary protein quantity/quality or stage of lactation (Jansen *et al.* 1986, Millican *et al.* 1987, Sampson *et al.* 1984c, Sampson *et al.* 1986, Chapter 3). Rates of liver FSR reported here (79 - 85 %/d), compare well with those of previous studies using the flooding dose technique in rats (70 - 80 %/d, Sampson *et al.* 1984; 85 - 103 %/d, Chapter 3) and mice (73 - 78 %/d, Millican *et al.* 1987). Although liver FSR appeared to be unaffected by nutrition or stage of lactation, in group H liver ASR (mg/d) was significantly increased from day 4 (1.76 mg/d) to day 12 of lactation (2.54 mg/d) as a result of considerable organ hypertrophy, while in comparison the feeding of diet L promoted a significant reduction in liver size and thus ASR. These observations support the conclusion that alterations in hepatic protein synthesis during lactation are determined by nutritional influences on liver size and protein content (Jansen *et al.* 1986, Sampson *et al.* 1984c). Despite the possible increase in liver size and total protein synthesis during lactation, alterations in hepatic protein metabolism, particularly the enzymes of the urea cycle, go some way to spare available amino acids for extra hepatic use (Barber *et al.* 1990, Naismith *et al.* 1987).

Muscle Protein Metabolism

Previous studies involving lactating rodents have suggested that well nourished dams, regardless of age/maturity, can satisfy their increased requirement for protein through a considerable elevation of feed intake and do not depend upon muscle protein as an endogenous nutrient source (Glore *et al.* 1985, Millican *et al.* 1987, Naismith *et al.* 1982, Chapters 2 and 3). The results of the current study support this principle and also reject the possibility that in well nourished dams muscle protein undergoes periods of depletion and repletion during lactation (Chapters 2 and 3). However, when such females are subjected to severe undernutrition or offered imbalanced diets (low protein/high energy) that suppress feed intake, they attempt to supplement their nutrient supply, and thus sustain milk production, by mobilising their endogenous protein reserves. Although the use of maternal protein can have a significant impact on lactational performance (Chapter 2), this influence is constrained by the metabolic limit of such reserves (Allison *et al.* 1965) and the effect of prior nutrition upon their repletion (Chapter 2).

In the current study, the significant loss of muscle protein from group L during lactation (133 mg), although supporting the above observations, appeared to occur rapidly during early lactation and the bulk of this loss was achieved by day 8, with little change occurring during the last 4 days (Fig. 6.4). These changes in muscle protein content reflect the previously reported pattern of carcass protein loss from similarly treated females (Chapter 4). Although the results from an earlier study suggested that both an increase in degradation and a decline in synthesis were involved in the loss of muscle protein during lactation (Chapter 3), the degradation rate was calculated assuming a constant rate of protein loss throughout lactation. This assumption has now been shown to be inaccurate and the rapid loss of muscle protein during early lactation would require a much higher rate of degradation (FDR).

The suggestion that the loss of muscle protein during early lactation is associated with a dramatic increase in FDR is further supported by the fact that although in the current study severe protein restriction from parturition resulted in a significant decline in muscle

FSR (3.29 - 2.03 %/d), this fall was not rapid and the greatest reduction occurred between day 8 and 12 of lactation (P=0.08) (Table 6.1). Using the average muscle FSR and protein contents (Fig.6.4) of group L between each slaughter point, the average muscle degradation rates were calculated and are shown in Fig. 6.5. Between day 2 and 4 of lactation, the average FDR of 13.0 %/d was considerably greater than the FSR (3.1 %/d) and promoted the loss of muscle protein at 34.9 mg/d. As lactation proceeded, both muscle FSR and FDR fell to rates which between day 8 and 12 of lactation were not substantially different. This decline in muscle FDR during the later stages of lactation is possibly part of the mechanism that prevents excessive catabolism of maternal protein during this period and by day 12 of lactation the decline in FDR could have resulted in the rate being lower than that of muscle FSR, and which would therefore allow the recovery of maternal protein mass. A similar mechanism has also been shown to be involved in the prevention of muscle protein loss during the early stages of starvation and protein restriction in growing animals (Millward *et al.* 1978). It is therefore apparent that a dramatic increase in muscle FDR is central to the rapid mobilisation of maternal protein during early lactation, while the decline in protein synthesis has a smaller and later role.

The results of the current study, although in agreement with those reported by Vincent *et al.* (1985) and the suggestion (Bryant *et al.* 1982) that in ewes the activity of muscle proteolytic enzymes and thus protein degradation may be increased during lactation, contrast with the conclusions of Swick *et al.* (1977) and observations of Champredon *et al.* (1990) and Baracos *et al.* (1991) that protein mobilisation during early lactation in goats is associated primarily with a decline in muscle protein synthesis. These latter studies only estimated rates of tissue protein synthesis at one point during lactation and were compared with those of non lactating controls, while the use of one infusion period also prevented the degradation rate from being calculated. Furthermore, since the change in muscle FSR during lactation was small and insignificant and the lactating females were in negative nitrogen

balance (-4.4 g/d) (Baracos *et al.* 1991), the use of additional slaughter points could have established whether alterations in muscle proteolysis also had a key role.

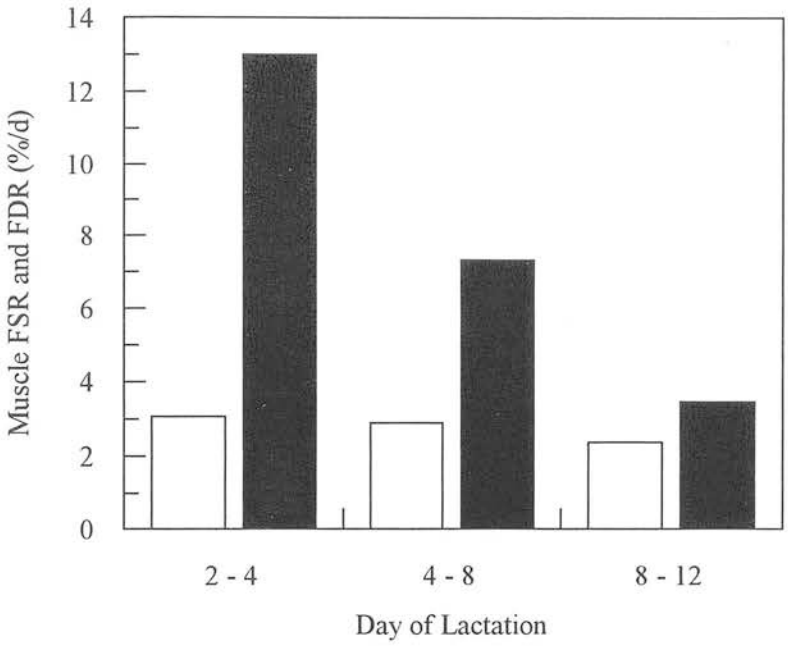

Fig. 6.5. Calculated rates of protein synthesis (FSR) (□) and degradation (FDR) (■) in the skeletal muscle (gastrocnemius) of females offered diet L during lactation. Fractional degradation rates were calculated using changes in the mean fractional synthesis rate and muscle protein content.

It might have been expected that this loss of muscle protein from mature females would be associated with an increase in degradation since the rate of muscle protein turnover (synthesis and degradation) in such females is extremely slow (Table 6.1) and substantial reductions in synthesis alone could not have promoted such a rapid rate of loss (Millward *et al.* 1976).

How muscle protein metabolism responds to alterations in nutrient supply is influenced by the muscles composition of oxidative and glycolytic fibres. The gastrocnemius muscle, a muscle of mixed fibre type profile, is an example of the majority of muscles in a rats body and in the current study the decline in it's FSR in response to protein restriction was reflected in a reduction of both the RNA content and activity. Such an association between

muscle RNA content and activity and the decline in gastrocnemius FSR has been previously reported (Baille *et al.* 1991) although muscles of different fibre composition (e.g. soleus) may alter their rate of synthesis through changes in RNA content alone (Pain *et al.* 1983).

Although in the protein restricted females the partitioning of amino acids away from the bodies musculature during lactation would have been encouraged by the associated hypoinsulinaemia (Williamson 1980) and reduced muscle insulin sensitivity (Burnol *et al.* 1987), the mechanism that stimulates the dramatic increase in muscle proteolysis remains to elucidated. The circulating ratio of insulin: corticosterone has been proposed to be involved in the control of protein catabolism during pregnancy (Naismith 1966) and corticosterone has opposing effects to insulin on muscle metabolism in rats, inhibiting protein synthesis (Southorn *et al.* 1990) and possibly stimulating degradation (Odedra *et al.* 1982). Muscle proteolysis, but not synthesis, is also stimulated through increases in intracellular concentration of prostaglandin E_2 during fever and sepsis (Goldberg *et al.* 1984), although changes in muscle prostaglandin activity during lactation are unknown.

In summary it can be concluded that when lactating rats are subjected to a period of severe dietary protein restriction, the rapid mobilisation of their endogenous protein reserves is associated with a dramatic increase in the rate of muscle protein degradation, with the decline in protein synthesis being slower and of less importance. Excessive protein catabolism during the latter stages of lactation is however prevented by a considerable fall in FDR, possibly below that of synthesis. The estimation of *in vivo* mammary protein synthesis by the flooding dose technique appears to be impaired in rats that have been previously milked using exogenous oxytocin, although rates of muscle and liver FSR appear to be unaffected. Further work into the influence of exogenous oxytocin on mammary protein synthesis and how this changes with variations in dose level is required before future combined studies of milk composition and mammary protein synthesis can be attempted.

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CHAPTER SEVEN

DISCUSSION and FUTURE WORK

GENERAL INTRODUCTION

The central theme to the work presented in this thesis has been concerned with enhancing our understanding of the partitioning of protein between sites of accretion and secretion in lactating rodents, with particular emphasis on the utilisation of maternal protein reserves under conditions of severe dietary protein inadequacy. The extent to which body protein stores can be used to support milk production under such conditions is ultimately influenced by a combination of interdependent factors which can be separated into two groups. The first is concerned with those factors that determine the availability of maternal protein and were therefore considered as the original objectives of this thesis, while the second is associated with the integration of this available endogenous protein supply with other nutrients in support of milk production.

1) Factors that determine the availability of maternal protein during lactation;

: Conditions associated with lactation that promote tissue protein loss
: Extent of protein reserve repletion
: Potential rates of maternal protein loss.
: Controlling mechanisms of tissue protein metabolism

2) The interaction between dietary and endogenous nutrients in support of milk production.

Whilst in the completion of this thesis I have investigated the effects of severe protein undernutrition on other aspects of maternal metabolism during lactation, including tissue Na^+,K^+-ATPase activity, rates of tissue protein synthesis (mammary gland, liver, gut mucosa), mammary gland functional integrity and milk composition, the majority of this discussion will concentrate on the two groups mentioned above. The effects of dietary protein restriction on the additional areas of maternal metabolism during lactation are covered extensively in the relevant chapters.

Conditions Associated with Lactation that Promote Tissue Protein Loss

The results of this study have shown that whilst mature females, suckling a large litter (12 pups), relied upon an increase in feed intake to support milk production when dietary protein was not limiting, under conditions of dietary protein inadequacy such an increase in feed intake was in some way prevented and dams were forced to catabolise their endogenous reserves of protein.

Since parity is thought to influence the priority given to milk production and thus the drive to utilise body protein by lactating females (Bruckental *et al.* 1989, Oldham *et al.* 1989, Young *et al.* 1985), the use of multiparous female rats for all experiments in this study allowed the loss of body protein during lactation to be attributed to the lactational stress imposed and variations in nutrient supply.

Numerous studies have suggested that not only are the reserves of body protein required to supplement an inadequate dietary supply, but that well fed lactating rodents also draw upon such reserves when lactational demand is high, for example to support the growth of a large litter (Kanto *et al.* 1980, Sainz *et al.* 1986a, Taylor *et al.* 1986). The results of this study however, did not support this latter proposal and suggested that, in a similar way to Friggens (1990), mature females offered high protein/high energy diets and suckling large litters, relied upon an increase in feed intake to allow milk secretion to provide sufficient nutrients for litter growth. While it is possible that the animals used in this study were of a lower "yield potential" and thus less willing to enter into a protein deficit than were the primiparous females used in the other studies, this seems unlikely since for dams offered the 215 gCP/kg DM diet in this study, the weight gain of a standardised litter between day 7 and 13 of lactation (Table 2.4) was in excess of the limited data reported by Taylor *et al.* (1986) for dams offered a high protein feed during the same period. Although the amino acid supply may not have been supplemented from muscle protein, their availability may have been

increased by sparing mechanisms associated with hepatic protein metabolism (Barber *et al.* 1990, Naismith *et al.* 1987).

During the current study, when lactating females were offered diets of a lower protein content (and protein:energy ratio) the ability to exhibit such an increase in feed intake throughout lactation was constrained, and this is in agreement with the results of similar studies using diets of inadequate protein quantity (Friggens 1990, Naismith *et al.* 1982) and quality (Sampson *et al.* 1986). On such diets, because of the imbalance in protein and energy yielding nutrients, which may be further exaggerated by the mobilisation of body fat, the constraint on feed intake is thought to prevent an excess of energy yielding nutrients which could not adequately be accommodated by milk production and the storage or oxidation of surplus nutrients (Naismith *et al.* 1982). Friggens (1990) hypothesised that a female's capacity to lose heat was ultimately responsible for constraining feed intake on such imbalanced diets, although this remains to be fully established. This position contrasts markedly with analogous situations in growing animals (Kyriazakis *et al.* 1990, Musten *et al.* 1974) who alleviate such a deficit in dietary protein by increasing feed intake and the consequent storage of surplus energy yielding nutrients.

In experiments E1, E3 and E4, the combination of suppressed feed intake and reduced dietary protein content, resulted in the mobilisation of body protein stores. Although the feed intake and litter growth of females offered the 215 and 150 gCP/kg DM diets were similar during early lactation (Fig. 7.1a, 7.1b), the supply of dietary protein from the 150 gCP/kg DM feed appeared to be insufficient to support this litter growth and required the catabolism of body protein to sustain milk production at the level of the females offered the improved protein supply. Subsequent alterations in dietary protein content, to 90 g/kg DM, and feed composition ultimately compromised nutrient supply further and promoted a greater demand on the endogenous protein stores ($P<0.10$), although the use of such reserves could not maintain litter growth at the level of dams offered the higher protein diets (Fig. 7.1b). The

results of this study further suggest that the content of high quality protein in diets offered to lactating rodents is an important determinant of body protein utilisation during lactation.

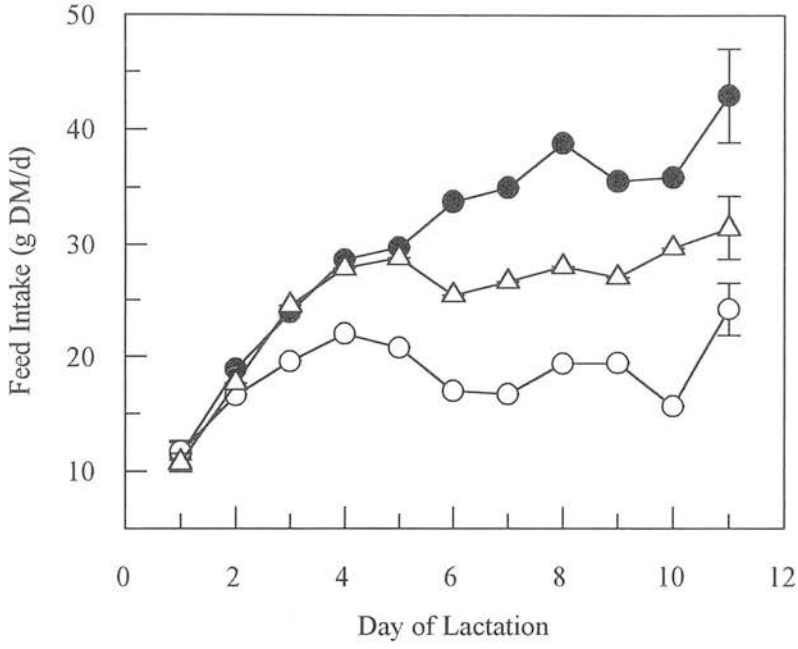


Fig. 7.1a. Daily feed intake (g DM) of females offered isoenergetic diets containing either 215 (●), 150 (△) or 90 gCP/kg DM (○) during lactation. Data are derived from experiments E3 (150 gCP/kg DM) and E4 (215 and 90 gCP/kg DM) and include data for all females offered the respective diets at each stage of lactation. Each point represents a mean and representative SEMs are included for days 1 and 11.

The loss of body fat during lactation is thought not to be obligatory but depends on the extent of reserve repletion at parturition (Garnsworthy 1988). The high energy diets offered during gestation in this study resulted in the considerable storage of body lipid during the latter stages of pregnancy (Fig. 2.2). Such an accumulation, in preparation for lactation, has been suggested to necessitate the mobilisation of fat during lactation (Naismith *et al.* 1982) and therefore return body fatness to levels that relate to the physiological state. Naismith *et al.* (1982) also concluded that the loss of such reserves is under hormonal rather than dietary control and, although the results from experiment E3 suggest that dietary treatment may have had some influence, the considerable loss of adipose tissue in experiments E1 and E3 tend to support this suggestion. The changes in adipocyte metabolism associated

with such mobilisation prevented the storage of surplus energy yielding nutrients in dams offered imbalanced (low protein/high energy) diets and thus contributed to the constraint on feed intake and lactational performance. As a result, the contribution of body fat to the energy supply for lactation was increased in dams offered the low protein compared to the high protein diets (30 vs 12%) (Table 2.3). In high yielding dairy cows the contribution of endogenous lipid to milk production is even more impressive and Wilson *et al.* (1988) have reported that during early lactation over 50 % of milk fat carbon is derived from the body fat reserves.

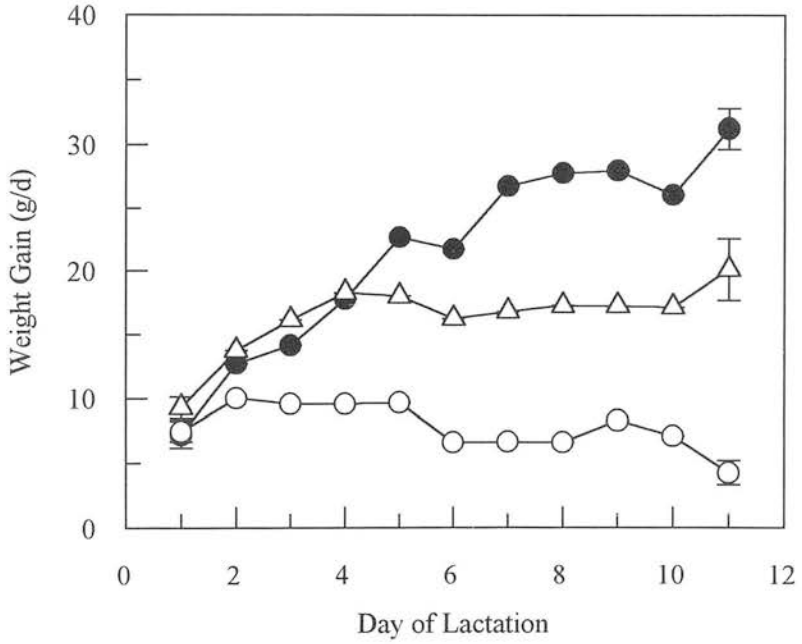


Fig. 7.1b. Daily litter weight gain (g) of females offered isoenergetic diets containing either 215 (●), 150 (△) or 90 gCP/kg DM (○) during lactation. Data are derived from experiments E3 (150 g CP/kg DM) and E4 (215 and 90 g CP/kg DM) and include data for all females offered the respective diets at each stage of lactation. Each point represents a mean and representative SEMs are included for days 1 and 11.

Extent of Reserve Repletion at Parturition

From the results of experiment E1 it can be concluded that variations in the extent of reserve repletion at parturition can have a significant effect on a lactating rodent's ability to maintain milk output under conditions of dietary protein inadequacy. However, when an

adequate supply of dietary protein was provided during lactation (215 gCP/kg DM) such variations in reserve repletion had little impact on lactational performance, and during lactation the litter growth of dams that were protein depleted during gestation was comparable to that of females considered to be "Fully" protein replete at parturition.

Protein reserves have been suggested to account for up to 25 % of body protein (Allison *et al.* 1965, Botts *et al.* 1979) and an apparent metabolic limit prevents excessive catabolism of such reserves in order to maintain the body's functional integrity. During the current study, females that were assumed to possess "Fully" replete protein reserves at parturition were capable of losing between 15 (E3) and 22% (E1) of body protein when subjected to severe protein restriction during lactation. This is close to, but a little less than the hypothesised metabolic limit. The variation in the extent of protein loss possibly reflects unavoidable variations in reserve repletion at the initial reference points in this study. Clearly the extent of reserve repletion at parturition is influenced by changes in maternal protein mass both before and during pregnancy, and the changes reported in experiment E1 (Fig. 2.1) that suggest body protein mass follows a biphasic pattern during gestation (Naismith *et al.* 1976), would possibly constrain the quantity of protein subsequently available.

Despite these possible variations in reserve repletion, the results of experiment E1 also confirm that females entering lactation without having previously suffered severe protein depletion, are subsequently better prepared to sustain milk production when dietary protein is limiting. During early lactation, "Fully" protein replete dams were capable of supporting a significantly better litter growth (45%) (Table 2.4) than those subjected to gestational treatments that severely depleted their protein stores. This improved lactational performance was not only the result of a greater quantity of endogenous protein but also the capacity to increase the intake of the limiting nutrient (protein), and these results are in agreement with those reported by Mahan *et al.* (1975) for first litter sows. While such reserves can have a dramatic impact on a dam's capacity for lactation, because of their limited size they cannot support milk production for long, or to the level of well fed females.

It seemed from the initial studies that in severely protein restricted dams, the changes in litter weight gain during mid lactation reflected the exhaustion of the available protein reserves and thus their limited capacity to sustain milk production. Subsequent studies confirmed that the metabolic limit of such reserves, exhibited by the day 12 body protein mass of groups HL_2 and LL_2 (E1) (Fig. 2.1), was reached shortly after day 6 of lactation (Figs. 4.6, 6.4) and therefore supported this conclusion. Following such a depletion, mammary gland metabolism depends primarily upon dietary protein supply.

Although body protein reserves can act as an important, but limited, buffer in times of nutritional adversity, this study has shown that the extent of their repletion at parturition is unimportant to subsequent milk production when adequate nutrition was provided and thus supports the conclusions of similar studies involving rats (Kliewer *et al.* 1987) and sows (Mahan *et al.* 1975). In addition, while achieving a comparable milk production to similarly well fed females (Fig. 2.3), such protein depleted dams were also attempting to replenish their body protein stores. Whether the response to a higher level of dietary protein would be an improved lactational performance and protein replenishment or whether, in a similar way to that reported by Botts *et al.* (1979), a shift in protein partitioning would result in sites of accretion receiving a greater proportion of dietary protein at the expense of milk production remains to be elucidated.

Despite the fact that the major organs (liver, mammary gland, G.I. tract) undergo reductions in size and protein content when dietary protein is limiting during lactation (Tables 2.6, 6.2, 6.3), their contribution to the overall protein supply is minor and the bulk is derived from carcass protein (muscles and skin). The limited use of protein from the major organs may have important implications in allowing functional protein to maintain tissue metabolism, although the use of this amino acid supply may become more important when muscle protein reserves are exhausted. Whereas the loss of whole body protein in this study did not exceed the suggested metabolic limit, the 34 % loss of gastrocnemius muscle protein in experiment E4 (Table 6.1) possibly suggests that individual muscles respond differently to such a

catabolic stimulus. Such a response may be influenced by the muscle's functional role (e.g. locomotion), although Galler *et al.* (1980) reported a reduced physical activity of protein restricted pregnant rats.

Potential Rates of Maternal Protein Loss

The results of this study have confirmed that in mature female rats suckling large litters (12 pups), the rate of maternal protein loss during lactation can be adjusted in response to variations in dietary protein inadequacy. It might be concluded that the rate of protein loss was strongly influenced by the difference between nutrient supply and demand.

Although the potential of protein reserves to support milk production depends on both the extent of reserve repletion and the rate at which amino acids are made available for metabolism, the majority of studies that have described their use by lactating rodents (Friggens 1990, Kanto *et al.* 1980, Naismith *et al.* 1971, Naismith *et al.* 1982) have failed to give an accurate indication as to the contribution of such reserves to the daily nutrient supply and how this may be influenced by dietary conditions.

The suggestion that the decline in milk output during mid lactation of dams subjected to severe nutritional stress resulted from the exhaustion of their body protein stores (Friggens 1990) has been confirmed by the current study, and under conditions of severe protein restriction (90 g CP/kg DM), body protein loss occurred rapidly during early lactation and resulted in an apparent depletion of reserves between days 6 and 9. The estimated fractional rate of mobilisation under such conditions, and assuming that protein reserves were exhausted by day 9 of lactation, approximates to 0.021 d^{-1} (1.01 g/d), although it might be expected that this rate would be closer to 0.028 d^{-1} (1.28 g/d, day 7) since little change in body protein mass occurred between day 6 and 9 in experiment E3 (Fig. 4.6). Applying a similar assumption to the 10.3 g protein loss by group HL_2 (experiment E1) (Table 2.3) under comparable dietary conditions would require a fractional rate of 0.038 d^{-1} (1.72 g/d). Such estimations of rapid mobilisation during early lactation, further confirm that the assumption

that protein loss occurs throughout lactation under similar dietary conditions would considerably under-estimate the maximal daily contribution of endogenous protein to milk production (Table 1.6), and such rates of loss are comparable with the maximal rates reported for female rats offered restricted access to a low protein diet for seven days during mid lactation (1.19 g/d) (Sainz *et al.* 1986b). Whilst these fractional rates of mobilisation were calculated in relation to the total maternal protein mass, it must be noted that such rates of loss would be considerably increased if the labile protein pool (25 % of body protein) is considered in isolation, and such rates would be increased to 0.084 d^{-1} (1.01 g/d) and 0.107 d^{-1} (1.28 g/d).

Although it might be expected that the muscle protein amino acid composition may not precisely match the mammary gland requirement and that such rapid rates of protein mobilisation would quickly deplete the limited reserves available, during early lactation endogenous protein can provide up to 40 % of the total protein supply (E3). Despite this important contribution to the nutrient pool, endogenous protein loss occurs at a slower rate than that of body fat. From the results of this study, it is apparent that body fat provides a continual supply of milk fat precursors throughout the 12 day lactation period, and fractional rates of loss can be calculated to be on average 0.052 d^{-1} (E1), and this compares to a similar rate estimated for cattle of 0.064 d^{-1} (Konig *et al.* 1979).

Not only does the dietary protein content influence the drive to utilise body protein by lactating rodents, it also determines the rate at which such protein reserves supplement the available nutrient pool and it might be suggested that the rate of loss is a function of the difference between dietary supply and nutrient demand. Whilst the feeding of the 150 g CP/kg DM diet promoted the loss of body protein in support of milk production, this mobilisation occurred throughout lactation at 0.01 d^{-1} (0.49 g/d), which was considerably slower than that of comparable dams offered the 90 g/kg DM diet. Such a variation in the rate of body protein loss is also supported by the calculated rates of mobilisation from Sainz *et al.* (1986b) and

Friggens (1990) (Table 1.6) that suggest the rate of loss during lactation is influenced by the extremes of nutritional inadequacy.

Such a capacity to vary rates of mobilisation in response to alterations in dietary protein supply, is likely to be part of an overall mechanism by which lactating females integrate signals of nutrient supply (diet) and demand (milk production/litter) and which operates through the regulation of muscle protein breakdown in an attempt to sustain milk secretion. The observation that the supply of endogenous protein (0.49 g/d) during early lactation allowed dams offered the 150 gCP/kg DM diet to support litter growth that was comparable to females consuming similar quantities of a higher protein feed (215 g /kg DM), lends some support to this suggestion. Although such a mechanism promotes the loss of considerably more muscle protein with further reductions in dietary protein supply, such rates of protein mobilisation cannot prevent milk output in these females from being severely impaired.

This regulation of protein mobilisation may also balance the short/long term cost and benefits of such body protein loss. Although under conditions of severe protein restriction (90 g CP/kg DM) litter growth would be curtailed without the initial support for milk production from the rapid utilisation of available protein reserves, such a loss of protein would, if allowed, soon threaten maternal protein integrity. It therefore seems reasonable that a metabolic limit is imposed on body protein loss, after which the lactational support of the litter is sacrificed. For dams offered the diet containing 150 gCP/kg DM and where such rapid rates of mobilisation are not required to support litter growth during early lactation, the fact that the endogenous supply of protein was apparently consistent throughout lactation limited the support that the body protein reserves provided for milk production during mid lactation and thus prevented a further improvement in feed intake and lactational performance during this period (Fig 4.2, 4.3). Although this had a short term effect of preventing an initial improvement in milk output, in the long term it allowed litter growth to be maintained for longer, although at a slower rate.

This ability to adjust the rate of muscle protein breakdown therefore provides lactating dams with a possible strategy for supporting milk production under variable conditions of nutritional inadequacy. While allowing lactation to continue during periods of deficiency, the rate of mobilisation and the extent of reserve repletion ultimately impose a limit to which such support can be maintained. Whereas rapid protein breakdown is essential for the short term support of lactation under severe conditions, the long term support of lactation by the slower rate of endogenous protein supply during less extreme periods of inadequacy, allows litter growth to be maintained until improvements in dietary supply can be possibly obtained.

Controlling Mechanisms of Muscle Protein Metabolism

Of particular importance to the theme of this thesis was the attempt to provide a better understanding of the alterations in muscle protein turnover involved in promoting the rapid mobilisation of protein reserves during lactation, and following experiments E2 and E4 considerable progress has been made. From the results of these studies it can be concluded that a dramatic increase in protein degradation is primarily responsible, while changes in synthesis have a much smaller and somewhat later role. While this has not been previously established for lactating rodents, although alterations in muscle protein degradation have been implicated as being part of the mechanism involved in promoting protein mobilisation (DeSantiago *et al.* 1991, Sainz *et al.* 1984), the confirmation that changes in FDR were ultimately involved might have been expected, since rates of muscle protein turnover are extremely slow in mature animals and thus reductions in synthesis alone would not be capable of promoting such a dramatic breakdown of protein (Millward *et al.* 1976).

While in severely protein restricted dams this shift in muscle protein turnover allows considerable quantities of endogenous protein to be released, such a balance of protein metabolism could, if allowed, quickly deplete muscle protein and eventually impair its functional integrity. The metabolic limit of body protein reserves prevents such excessive

catabolism and muscle protein turnover must therefore be regulated to accommodate such a limit. However, rather than an increase in synthesis being involved, from this study it is clear that such excessive catabolism is prevented by a fall in degradation to rates that are eventually below that of synthesis (Fig. 6.7).

These results have highlighted the necessity of considering the mechanism involved as one which can quickly "switch on" protein degradation upon demand but then "switch off" such catabolism to prevent excessive muscle damage. Although the components of such a "switch" remain to be elucidated, it is clear that muscle proteinase enzymes are involved, of which the calpain/calpastatin system appears to be of particular interest since the enzyme's activity can be regulated by (i) conversion between its active/inactive forms and (ii) an intracellular inhibitor (Higgins *et al.* 1988). Whether such catabolism is also non-specific, as suggested by Swick *et al.* (1977), or whether particular subcellular protein fractions are targeted, as under anabolic stimulus (Adeola *et al.* 1992), needs clarification.

Although rates of muscle protein turnover were not estimated in dams offered the 150 gCP/kg DM diet during lactation, it might be thought that the changes involved in the slower rate of muscle protein release would be qualitatively similar to those of the severely restricted females although quantitatively less dramatic.

While, from the results of this study, alterations in muscle protein synthesis are not thought to be particularly important in promoting the rapid mobilisation of protein during early lactation, Mayel-Afshar *et al.* (1983) have suggested that during the catabolic phase of gestation the decline in muscle protein synthesis may act to spare amino acids from maternal use for foetal and placental development. However, the ability of protein depleted females to partition an improved supply of dietary protein towards the replenishment of such reserves during lactation depends on the capacity to enhance the muscle's anabolic processes and a similar mechanism has been reported to be involved in the gain of body protein during the anabolic phase of gestation (Mayel-Afshar *et al.* 1982). In addition, since the musculature of the body, by its sheer volume, makes a substantial contribution to whole body protein turnover

and thus maintenance energy expenditure (Baldwin *et al.* 1980), reductions in muscle protein metabolism, including the metabolic support processes (Na^+,K^+-ATPase), would spare energy utilisation in favour of other body tissues. However, in the well fed dams of this study, whose maintenance energy requirement may be increased by almost 25 % during lactation (Canas *et al.* 1982), no such energy sparing ability was observed. The proposed link between rates of muscle protein synthesis and Na^+,K^+-ATPase activity (Vandenburgh *et al.* 1981, Adeola *et al.* 1989) is however, supported by the results of this study. The decline in muscle protein synthesis during lactation in severely protein restricted dams was reflected in a reduction in Na^+,K^+-ATPase dependent respiration (Table 3.6), which may have favoured the reduced uptake and utilisation of amino acids by muscle tissue. Whilst the increased activity of this enzyme in the mammary gland of well fed females during lactation (Chapter 3) reflects the need for an enhanced support of mammary protein synthesis, also reported in this study (Table 3.7), no such change was reported in the other tissues studied. However, the considerable organ hypertrophy (Table 2.6; liver, mammary gland, gut) associated with lactation in well fed females will have ultimately increased the total respiration, and energy expenditure associated with this membrane transport system in these tissues.

Although the experiments undertaken for this thesis have gone some way to establishing the probable mechanism involved in the utilisation of maternal protein reserves during lactation, they could not provide any information as to the associated homeorhetic control signals. Whilst such partitioning would be favoured by the reduced muscle insulin responsiveness (Burnol *et al.* 1987) and hypoinsulinaemia (Williamson 1980) associated with lactation, information concerning the endocrine signals that are responsible for promoting the dramatic increase in muscle proteolysis during lactation is extremely limited. Whether such a shift in muscle protein metabolism was influenced by an increase in serum coricosterone, and thus the cortisol/insulin ratio, that has been reported to occur in protein restricted lactating rodents (Kliewer *et al.* 1987) and which has been proposed to be involved in promoting muscle protein catabolism in pregnant rats (Naismith 1966), remains to be established.

Furthermore, how serum hormone levels, tissue responsiveness and intracellular mechanisms are integrated to control the "on/off" switch of muscle protein catabolism deserve further attention.

From the above, it is apparent that by addressing the original objectives of this study, the work presented in this thesis has provided an important description of how severely undernourished females utilise their body protein reserves in support of lactation. Furthermore, it has improved our understanding as to the extent to which such protein reserves can sustain milk output and the changes in muscle protein metabolism involved in this partitioning.

Although the use of body protein reserves by severely undernourished dams is often considered in relation to their short term contribution to the maintenance of milk secretion, this supply of protein may have other less well established functions. One such long term role, appears to be the maintenance of a functioning mammary gland during early lactation, which allows dams to quickly enhance their lactational performance in response to improvements in dietary supply. While observations from earlier studies (Peart 1970, Robinson *et al.* 1979) might suggest that such a capacity would be limited by eventual mammary gland regression, which in this study appears to coincide with the depletion of the tissue reserves (Chapter 4), this remains to be confirmed.

Whereas several studies have demonstrated that inadequate maternal intakes of protein can impair both milk quantity and quality in rodents, the work in this thesis has extended such investigations to possible variations in this effect throughout lactation. This study has shown that while a supply of endogenous protein can alleviate some of the affects of dietary inadequacy on milk yield, the same is not true for milk quality and the protein content was reduced throughout lactation. However, of perhaps more importance to dams offered low protein/high energy feeds is their apparent capacity to dispose of more energy in milk by increasing its lipid content. In dams supposedly constrained by their ability to dispose of surplus energy as heat (Friggens 1990), this ability may help overcome the imbalance of

protein and energy yielding nutrients, that are derived from dietary and endogenous sources, and thus reduce the requirement for heat disposal. While this could possibly improve feed intake, its impact is limited by milk yield and thus the supply of protein. Whilst equations that have been developed to estimate the milk production of lactating rats from litter weight gain and litter weight (Sampson *et al.* 1984b) may be used in providing useful information on milk yield of well fed dams, the results of this study have indicated that under conditions which may induce changes in both milk yield and composition (protein restriction, cafeteria feeding) their use is questionable.

This study has clearly demonstrated the influence that body protein reserves can have on both the short and long term lactational performance of dams offered low protein/high energy feeds, and how this influence may be altered by variations in both the extent of reserve repletion and rate of protein release. However, rather than being considered in isolation, the importance of these reserves to such females should also be related to their interaction with other dietary and endogenous nutrients to support milk production and possibly limit the impact of a nutrient imbalance.

The Interaction Between Dietary and Endogenous Nutrients in Support of Milk Secretion.

Amino acids are not only used by the mammary gland in the synthesis of milk proteins, they are also required for the maintenance of mammary integrity and the complement of enzymatic proteins involved in milk biosynthesis. The volume of milk secreted is primarily determined by the mammary gland's lactose production and in many mammalian species, due to their common secretory mechanism, milk protein and lactose concentrations are interrelated (Jenness *et al.* 1970). Since it has been suggested that an alteration in the secretion of one or more milk components may affect milk yield (Davies *et al.* 1983), it might be assumed that milk secretion would be reduced by protein inadequacy (quantity/quality).

Whilst a reduction in dietary protein supply would therefore have a deleterious effect on milk production and consequently litter growth, in terms of maternal metabolism it also

limits one of the major mechanisms of energy disposal by lactating females and increases the surplus of energy yielding nutrients. The low protein/high energy feeds used in this thesis would further enlarge this energy surplus, despite a limited increase in energy output following an alteration in milk composition (Chapter 5). Under such conditions, this metabolic embarrassment is unlikely to be eased via the storage of fat, but further exacerbated by the considerable mobilisation of the adipose reserves accumulated during gestation. The inevitable consequence of this would be an inability to dispose of surplus energy yielding nutrients and thus a possible limitation on nutrient intake.

Whilst energy disposal via milk output and lipid accumulation are limited by the supply of protein and adipocyte metabolism respectively, the release of heat following nutrient oxidation provides a further opportunity to alleviate this metabolic embarrassment. However, following the discussion of Blaxter (1989) it is apparent that animals exhibit a ceiling to their ability to dissipate heat (Heat Capacity) and this appears to be influenced by a number of factors including environmental temperature. Since heat is also released as a consequence of normal metabolic processes (maintenance, milk production) the capacity of lactating females to dispose of surplus energy yielding nutrients through heat loss is therefore constrained. This limit has been proposed (Friggens 1990) to be responsible for the suppressed intake and hence lactational performance of dams offered imbalanced feeds (protein:energy). Although this hypothesis appears to be logically sound, it remains to be verified.

Although milk production depends on a supply of nutrients that are derived from dietary and endogenous sources, the contribution of the different endogenous nutrients (fat and protein) may have contrasting effects in protein restricted females. Whilst the supply of endogenous energy can compound the problem of surplus energy disposal when protein supply is limiting, the use of body protein reserves is capable of improving lactational performance under such conditions. In supporting this improved milk output and hence litter growth, this endogenous protein supply may go some way to alleviate the imbalance of protein and energy yielding nutrients and in doing so allow a further improvement in protein intake and thus milk

secretion. Whereas the extent of reserve repletion and rate of protein release have been shown to influence this ability, how lactating females combine such variations with other nutrients in support of lactation needs to be clarified.

In an attempt to highlight such nutrient interactions, a selection of simple nutrient balances have been constructed at various stages during lactation for dams offered the 215, 150 and 90 gCP/kg DM diets (Figs. 7.2 - 7.7). In such descriptions, the quantities of nutrients (exogenous, endogenous) available to the mammary gland are used to predict the maximum milk yield possible, from the first limiting nutrient, and this is then compared with the recorded litter growth. The relationship between the heat capacity and heat production of such females is also considered in relation to the possible constraint on feed intake. While this simple approach to such a complex subject will have obvious inadequacies, it does provide a useful tool for describing possible interactions. Although a nutrient balance was constructed for all diets during early lactation (day 3), the effects of such dietary treatments on subsequent lactational performance and maternal protein loss influenced the stage of lactation chosen for the second nutrient balance. For dams offered the 215 and 90 g CP/kg DM feeds, a nutrient balance was constructed during the latter stages of lactation, day 9 and 11 respectively, because the high protein diet allowed feed intake and litter growth to increase throughout lactation, while for the severely protein restricted dams their supply of endogenous protein is thought to have been curtailed by this stage of lactation. However, for dams offered the 150 g CP/kg DM diet, a second nutrient balance was constructed for day 6 of lactation to investigate the possible reasons behind the constraint on feed intake and litter growth during this period. The assumptions and equations used in the construction of such nutrient balances are described in Appendix 2. Whilst an animal's heat capacity can be calculated from the equation described by Blaxter (1989), the equation used here was that derived by Friggens (1990) since it has been shown to estimate a rodent's heat capacity as being comparable to their measured heat loss (Brody *et al.* 1938).

In the development of a nutrient balance for dams offered the high protein/high energy feed during early lactation (Fig. 7.2), the use of dietary carbohydrate for maternal maintenance was restricted to ensure that dietary protein was limiting milk yield. Despite this, it is apparent that whatever nutrient was made first limiting, the supply of dietary protein would allow milk yield to be more than sufficient to meet the litter demands for growth, without the need to deplete body protein. It is also interesting to note that at this level of intake and milk production, the estimated heat production/loss was considerably lower than the dam's heat capacity and therefore suggests that this was not involved in the regulation of feed intake.

During this period, despite the difference in the protein:energy ratio of the 150 and 215 gCP/kg DM diets, their intakes, of both dry (Fig. 7.1a) and fresh (33.0 and 32.1 g/d) matter, were similar and within the range reported for the same period in earlier studies (Friggens 1990, Glore *et al.* 1985, Moore *et al.* 1984, Naismith *et al.* 1982). Since the relationship between heat production and heat capacity does not appear to be involved in controlling the intake of the 150 gCP/kg DM feed (Fig. 7.3), and that a higher dietary protein concentration (300 gCP/kg OM) was able to promote a litter growth during this period (21.0 g/d, 13 pups; 1.6 g/pup/d, Friggens 1990) that was considerably higher than that of dams offered the 150 (16.0 g/d; 1.3 g/pup/d) and 215 g CP/kg DM diets (13.6 g/d; 1.1 g/pup/d) in this study (Fig. 7.1b), it might be suggested that during early lactation the consumption of such diets is regulated by other mechanisms, but not the litters suckling demand for nutrients. Whilst the intake of such females may have been regulated by their potential for milk production, the increase in gut capacity during lactation may have occurred at such a rate as to impose a physical limitation on feed intake.

Although the litter growth of dams offered these diets was comparable during this period (Figs. 7.1b, 7.2, 7.3), the lower protein content of the 150 g CP/kg DM diet resulted in such females receiving a dietary protein supply that would have been insufficient to meet the demand for litter growth. Under such conditions, body protein reserves were used in an

attempt to sustain milk secretion, and at a rate of 0.49 g/d this endogenous protein supply appears to have been capable of supporting such litter growth when the calculated milk yield and litter requirements are compared (Fig. 7.3). Whilst the litter growth and thus milk production of these groups are thought to be comparable, their respective nutrient balances indicate an apparent discrepancy in milk yield. Although this discrepancy may have arisen from an under-estimation of the milk produced by the group offered the 150 gCP/kg DM diet, it is more likely to have resulted from an over-estimation of that of the group offered the 215 gCP/kg DM diet. One possible discrepancy in this milk yield estimation is that no account is made of the protein required for the considerable organ hypertrophy reported for such females (3.5 g/12d; Table 2.5, 2.6), the bulk of which may also be required during the early period of lactation (Table 5.3). The importance of such a protein requirement to the discrepancy in milk yields may be limited however, since for dams offered the 150 gCP/kg DM diet in experiment E3 no consideration was given to the small gain in their mammary gland protein content between day 1 and 3 of lactation (Table 4.4). Alternatively, the milk yield of dams offered the 215 gCP/kg DM may have been over-estimated by the assumption that protein was the first limiting nutrient (Fig. 7.2), which may not have been the situation *in vivo*.

Whilst the combination of dietary and endogenous energy yielding nutrients in the group offered the 150 gCP/kg DM feed necessitated the disposal of substantial quantities of surplus energy as heat, this level of heat production may have been reduced by an increase in milk energy output following alterations in milk composition, and in doing so further increased the difference between the heat production and heat capacity. If this difference provides the potential for an increased feed intake, the loss of body protein in moderately restricted females further supports the suggestion that intake was constrained by alternative mechanisms and that the rate of protein loss may have been influenced by a combination of dietary protein content and litter demand.

Further reductions in the dietary protein:energy ratio to 90 gCP/kg DM, resulted in an even more dramatic imbalance of dietary and endogenous nutrients. The dietary protein

content ensured that protein was severely limiting milk production, while the contribution of dietary and endogenous lipid to the energy surplus following milk secretion would necessitate the disposal of heat at levels that would approach the heat capacity and thus, in tandem with other possible mechanisms, prevent further improvements in intake. In an attempt to compensate for such a dietary imbalance and thus sustain milk output, dams were forced to draw upon their reserves of protein, although at a rate that would reach the metabolic limit earlier than in dams offered the 150 gCP/kg DM diet. Despite this, their estimated milk yield (Figs. 7.4a, 7.4b) could not be maintained at the level of the better fed females (Figs. 7.2, 7.3).

The nutrient balance constructed for such protein restricted females during early lactation (Fig. 7.4a), also describes the maximum milk yield and heat production possible if an endogenous supply of protein was unavailable and suggests that while the supply of dietary protein would initially limit milk yield, the need to dispose of heat above the theoretical heat capacity would require feed intake and hence milk production to be forced down in a manner that reflects the hypothesis proposed by Friggens (1990). Such a scenario further demonstrates the importance of body protein reserves to females subjected to severe protein restriction, not only for the provision of an additional supply of protein but also in allowing an improved nutrient intake and thus lactational performance.

It has already been suggested that the estimated rate of body protein mobilisation can vary depending upon when these reserves are assumed to become deplete (days 6 -9), and therefore Fig. 7.4 describes the nutrient interactions at two calculated rates of protein loss (Figs. 7.4a, 7.4b). While the procedures used in the development of these nutrient balances may be over simplified, they not only demonstrate that the extent of improvement in milk output depends on the supply of endogenous protein, but also at the faster rate of loss, the nutrient interactions allowed a milk production and heat loss that were close to the calculated litter requirements and heat capacity, and this supports the suggestion that this could be approaching the rate of loss occurring *in vivo*. Whatever the rate of mobilisation, it might be

concluded that the drive to utilise body protein is strongly influenced by the extremes of dietary protein inadequacy.

Whereas the relationship between a female's heat loss and heat capacity does not appear to be responsible for regulating the intake of the other two feeds at this stage in lactation (Figs. 7.2, 7.3), its importance is enhanced when the necessity to lose heat is amplified by the balance of nutrients provided by the 90 gCP/kg DM diet and tissue mobilisation, although this was eased by the increased milk fat content of such females (Fig. 5.6). If this relationship is involved in preventing an excessive and undesirable nutrient imbalance, an improvement in feed intake will only occur when either milk output is improved by an enhanced protein supply, as shown by group L/H in experiment E3, when the catabolism of body fat was reduced or even reversed, or when there is a fall in the environmental temperature.

Although the balance between dietary and endogenous nutrients is extremely influential on a female's milk output during early lactation, for dams that depend on a supply of endogenous protein for milk production, the maintenance (or removal) of this supply has a distinct impact on their lactational performance as lactation proceeds.

For females offered the high protein/high energy feed (215 gCP/kg DM) and who thus did not catabolise body protein during lactation, their capacity to increase feed intake and thus lactational performance throughout lactation confirmed the expectations derived from earlier studies. The nutrient balance developed for such females on day 9 (Fig. 7.5) indicates that their nutrient supply allowed a milk production and thus litter growth that was considerably greater than that on day 3 (Fig. 7.2). Despite a considerable supply of body fat, the combination of feed intake and diet composition resulted in a balance of nutrients in which fat was first limiting, and as a consequence of its synthesis from carbohydrate there was little need to oxidize surplus energy yielding nutrients. Since the heat production of such dams appeared to be much lower than their heat capacity, it might be suggested that the feed intake of these animals was not constrained by this relationship and as with earlier in lactation,

would have been associated with alternative mechanisms. The increase in milk production during lactation in these females may be assumed to have been associated with an elevation in mammary gland metabolism, and the results for protein synthesis and Na^+,K^+-ATPase activity (Table 3.7) reported in this study support such an assumption. Although in this study an attempt was made to identify the time-course of such changes in mammary protein synthesis, the impaired rates of synthesis estimated, using the flooding dose technique (Garlick *et al.* 1980), immediately after exogenous oxytocin had been used as part of the milking procedure applied to the same female (Chapters 5 and 6) prevented any satisfactory progress from being made.

However, such an ability to increase feed intake substantially was not available to females subjected to moderate dietary protein restriction (150 gCP/kg DM), and feed intake and lactational performance were constrained around day 4. From the nutrient balance constructed for day 6 (Fig. 7.6), the possible reasons behind this constrained performance may be explained . Although intake and the calculated milk yield were slightly higher than those described for day 3 (Fig 7.3), protein remained the first limiting nutrient despite the continuous endogenous supply (0.49 g/d). However, as a result of this and the imbalanced dietary composition, the quantities of dietary and endogenous energy yielding nutrients disposed of by oxidation were increased. Under these conditions, and assuming no change in milk energy output, the total heat production of such females was close to their theoretical heat capacity, which would ultimately limit further heat loss and nutrient oxidation. Thus in contrast to earlier in lactation, intake and thus lactational performance were possibly constrained by the limited ability to dispose of the balance of nutrients derived from dietary and endogenous sources.

It is interesting to note that although the supply of endogenous protein allows females to achieve a lactational performance that would not be permitted from the diet alone, the rate of mobilisation also acts to impose a limit to the possible improvements in feed intake and milk output. While the endogenous protein supply cannot be maintained beyond the

metabolic limit, an increased rate of mobilisation could, for a limited period, promote an improved lactational performance through an increased dietary and endogenous protein supply, and a rate of 1.0 g/d could promote a milk yield close to 39 g/d without the relationship between heat loss and heat capacity being compromised. This inability to increase the rate of protein loss, despite the apparent constraint on performance and the availability of maternal protein, therefore suggests that the major influence on body protein loss is the extent of dietary protein restriction.

At the rate of protein mobilisation required by dams offered the 90 gCP/kg DM diet during early lactation, the rapid depletion of the available protein reserves would ensure that during the second week of lactation milk output was supported by dietary protein supply alone and therefore severely reduced. However, when a nutrient balance was constructed towards the end of this period (day 11; Fig. 7.7), it could not satisfactorily describe the provision of nutrients for milk production or the extent of nutrient oxidation.

One major difficulty was that the calculated milk yield could not support the estimated litter growth. Although it is possible that the litter requirements had been over-estimated, it is more likely that litter growth was supported by an improved milk output promoted by an increase in available protein (0.4 g). Although such an input of protein may have been derived from partially replete protein reserves, it might be thought that at this stage of lactation this would be extremely limited and variable between individuals. Of perhaps more importance is the supply of tissue protein, which may be as much as 0.2 g/d, that may be released during organ regression in these females at this stage of lactation, while the reduction in amino acid metabolism by maternal tissues (liver) can act as a sparing mechanism in favour of milk production (Barber *et al.* 1990, Naismith *et al.* 1987). If the litter requirements are considered to be reasonably accurate, it must be assumed that by a combination of mechanisms, sufficient protein was made available to the mammary gland in order for the required milk yield to be achieved.

In addition to this, due to the excessive quantities of dietary and endogenous energy yielding nutrients, the nutrient balance estimated that the level of heat loss required was considerably greater than the female's heat capacity at this stage of lactation. While the previously described improvement in milk yield and a possible increase in milk fat content would go some way to alleviating the nutrient surplus, a reduction in the supply of endogenous energy would possibly provide the most important mechanism occurring *in vivo*. Although the total removal of the body fat supply would reduce the heat loss below that of the heat capacity, this is perhaps not necessary and possibly a combination of these mechanisms may be involved.

Despite the obvious errors and inadequacies associated with the construction of a nutrient balance under these conditions, it is apparent that without the supply of endogenous protein, the severely protein restricted dams were forced to reduce their feed intake and lactational performance below levels that they previously enjoyed.

While such nutrient balance studies provide a useful description of the partitioning of protein in support of milk production, such partitioning during lactation may be also influenced by the extent of protein reserve repletion at parturition (Botts *et al.* 1979). Group LH (Experiment E1) achieved a litter growth and feed intake that was comparable with dams offered the same feed but who had not been previously protein depleted, while attempting to replenish their depleted protein reserves (not significant). Although the reduced maintenance protein requirements for such protein depleted females (-0.07 g/d) could enable some degree of protein repletion, of perhaps more importance is an improvement in the efficiency of dietary protein utilisation (Barber *et al.* 1990, Naismith *et al.* 1987). How the mechanism involved in this protein partitioning would accommodate an improvement in dietary protein supply remains to be elucidated.

Although the calculations and assumptions used to describe the possible interaction between dietary and endogenous nutrients were crude, they clearly demonstrate the importance of the available nutrient balance to a lactating female's ability to support milk production.

Such calculations confirm that on a well balanced feed (high protein/high energy), an increasing milk output can be supported by an enhanced feed intake and the interaction of dietary and endogenous nutrients, while feed intake is not constrained by any metabolic embarrassment but by other mechanisms possibly associated with gut capacity. In contrast to this, when the diet offered is imbalanced (low protein/high energy), lactating females are forced to utilise their own body protein reserves, although the capacity of such an endogenous protein supply to maintain milk secretion is influenced by the severity of the dietary imbalance. Under such conditions of protein inadequacy, the interaction between surplus dietary and endogenous energy yielding nutrients may also have an important influence on the level of feed intake because of the hypothesised limit to heat disposal. While the extent of the dietary imbalance appeared to influence the rate of endogenous protein loss, and thus the length of time such reserves could support lactation, these nutrient balance studies have further confirmed the importance of such an endogenous supply to protein under-nourished females, not only through the provision of additional protein but also because of their ability to allow an enhanced feed intake.

This discussion has attempted to provide not only an explanation as to the factors involved in determining the availability of the body protein stores and their importance to females subjected to severe protein restriction during lactation, but also how such an endogenous protein supply allows a lactating female to utilise her balance of available nutrients in support of milk production. While this study has considerably improved our understanding as to the importance of this protein supply and how its influence is affected by variations in reserve repletion, a number of additional questions have been raised which as yet remain unanswered.

FUTURE WORK

From the experiments undertaken in pursuit of the objectives established at the outset of thesis, a number of important and interesting aspects of the control of protein

partitioning have been mentioned, although as yet the available information on these topics appears to be limiting. To improve our understanding of the mechanisms involved in the partitioning of protein between sites of secretion and accretion further effort into the following areas is required:

1) The effect of variations in litter demand (litter size) on both the loss and rate of loss of body protein under conditions of dietary protein restriction during lactation: Reductions in the litter size of well nourished dams during mid lactation results in a dramatic fall in milk production and feed intake (Grigor *et al.* 1983). Whilst such variations in litter demand would be of obvious benefit to severely protein restricted dams, how such variations influence the utilisation of body protein reserves when dietary protein is limiting during lactation remains to be elucidated.

2) The effect that the extent of protein reserve depletion at parturition has on the partitioning of available protein during lactation: Botts *et al.* (1979) suggested that the partitioning of feed protein between sites of secretion and accretion during lactation may be influenced by the extent of protein reserve depletion and dietary protein level since protein depleted dairy cows, offered a high protein ration, partitioned a greater proportion of feed protein towards the replenishment of protein reserves at the expense of milk production. Although it has been suggested that females of group LH (E1) in this study had attempted some degree of reserve repletion, this did not occur to any great extent and their lactational performance was not compromised in comparison to group HH. Whether the extent of this repartitioning in protein depleted females would have been improved by increasing the dietary protein content, but at the expense of milk production is at present unknown. In addition, how variations in the extent of reserve depletion affect such re-partitioning needs to be addressed.

3) The intracellular mechanisms that allow muscle protein degradation to be "switched" on and off: The results of this, and other studies, suggest that the availability of the body protein reserves is constrained by a metabolic limit that prevents excessive catabolism and thus helps maintain the body's functional integrity. While the rapid mobilisation of muscle protein during early lactation involves a dramatic increase in the breakdown of muscle protein that will be associated with the activity of muscle proteinase enzymes, the activity of such enzyme systems needs to be closely regulated to prevent excessive catabolism. The mechanism that permits such protein catabolism to be "switched" on and off during lactation remains to be established.

4) Factors that control the catabolism of muscle protein during lactation: Although it has been suggested that the utilisation of the body protein reserves has the capacity of being "switched" on and off to allow the rapid mobilisation of protein and the prevention of excessive catabolism respectively, the factors that control such a mechanism are at present not well understood. Whilst the hormonal control of muscle protein turnover in growing animals has received considerable attention, a similar situation does not apply to lactating animals. Furthermore, the extent to which the control of such muscle protein metabolism is influenced by circulating levels of hormones, tissues responsiveness or intracellular mechanisms deserves further attention.

5) The possible subcellular fractions of muscle protein that are targeted: Whilst Swick *et al.* (1977) suggested that body protein reserves are not made up of any specific storage polypeptide, several studies have indicated that individual subcellular protein fractions may respond differently to dietary and anabolic stimuli (Adeola *et al.* 1992, Millward 1970, Rikimaru *et al.* 1980). Whether the rapid mobilisation of muscle protein during lactation is at the expense of any particular subcellular protein fraction or that all muscle proteins are equally susceptible to degradation remains to be established.

6) The application of modelling techniques to describe the interaction between dietary and endogenous nutrients in support of milk production: While the nutrient balances that were constructed in this thesis were useful for describing the importance of the available nutrient balance to a lactating females ability to support milk production, they were crude and from the data accumulated in this and earlier studies (Friggens 1990) it should be possible to develop appropriate models that would describe and predict how females utilise available nutrients (exogenous and endogenous) in support of lactation.

CONCLUSIONS

1) The capacity of the body protein reserves to support milk production under conditions of severe dietary protein inadequacy was significantly affected by the extent of reserve repletion at parturition, and during early lactation the litters of "Fully" replete dams grew considerably more (45 %) than those of previously protein depleted females. In this study, dams that were assumed to have been "Fully" protein replete at parturition lost between 15 and 22 % of body protein during lactation, although the use of such reserves could not maintain milk production at the level of well fed dams. Reserves of protein are primarily associated with the tissues of the carcass (muscle and skin) and only a small amount of protein in the major organs was mobilised.

2) Prior depletion of the tissue protein reserves during gestation had no significant effect on subsequent lactational performance when an adequate supply of dietary protein was provided.

3) In lactating rats suckling large litters, the rate of maternal protein loss during lactation varied with the extent of the nutritional (protein) inadequacy. In "Fully" replete females such rates of loss ranged from 0 g/d on a 215 gCP/kg feed to 0.49 g/d (0.01 d^{-1}) and 1.01 g/d (0.021 d^{-1}) on 150 and 90 gCP/kg DM feeds respectively. The rate of body protein loss appears to have been influenced by the difference between nutrient supply and demand.

4) Lactating rats subjected to a period (5 day) of dietary protein restriction during early lactation were able to improve their lactational performance when the dietary protein supply was increased. The maintenance of the mammary cell population during this period of restriction favoured such an improvement in milk production. Whether such an increase would have been exhibited if these dams had been subjected to a prolonged period of restriction, especially after considerable mammary regression had occurred, is unknown.

5) The rapid mobilisation of maternal protein reserves during lactation was associated with a dramatic increase in muscle protein degradation (3. 5 - 13.0 %/d), while changes in the rate of synthesis were somewhat smaller and slower (3.3 - 2.0 %/d), and therefore of less importance.

6) The use of exogenous oxytocin as part of the milking procedure applied to lactating rats impaired the rate of *in vivo* mammary protein synthesis that was estimated using the flooding dose technique, while rates of muscle and liver protein synthesis were unaffected.

7) Severe dietary protein restriction during lactation not only reduced the milk production of lactating rats but also had a significant affect on milk composition (quality), reducing milk protein (90.7 vs 83.9 mg/g) and increasing milk fat (166.8 vs 205.5 mg/g). Under these conditions such alterations in both milk quantity and quality invalidates the use of milk yield prediction equations that were developed for well nourished dams.

Assumptions and Calculations Used in the Development of Nutrient Balances for Dams Offered the 215, 150 and 90 g CP/kg DM Feed.

a: The digestibility of feed and milk protein; 0.95 (Radcliffe *et al*. 1978)

b: The digestibility of feed fat; 0.97 (Chudy *et al*. 1969).

c: The digestibitlity of feed carbohydrate; 0.95 (Radcliffe *et al*. 1978)

d: Maintenance protein requirement g/d; $10*(0.07^{-0.27})$*(Body Protein) (Emmans *et al*. 1988, Friggens 1990))

e: Efficiency of protein use for maintenance and growth; 0.85 (McDonald *et al*. 1981)

f: Maintenance energy requirement kJ/d; $1630*(0.07^{-0.27})$*(Body Protein) (Emmans *et al*. 1988).

g: Efficiency of carbohydrate use for maintenance; 0.95 (McDonald *et al*. 1981)

h: Efficiency of fat use for maintenance; 0.97 (McDonald *et al*. 1981)

i: Efficiency of protein use for milk protein synthesis; 0.82 (Baldwin *et al*. 1968, 1980) .

j: Efficiency of fat use in milk fat synthesis; 0.89 (Baldwin *et al*. 1980)

k: Efficiency of carbohydrate use for lactose synthesis; 0.95 (Baldwin *et al*. 1980)

l: Heat production from milk protein synthesis; 16.7 kJ/g (Friggens 1990)

m: Heat production from milk fat synthesis; 4.4 kJ/g (Friggens 1990)

n: Heat production from lactose synthesis; 0.9 kJ/g (Friggens 1990)

p: Heat capacity; (1480.1 - 28*(temp ^{o}C))kJ/kg$^{0.75}$/d (Friggens 1990)

q: Supply of endogenous lipid

r: Efficiency of carbohydrate conversion to fat; 0.3 (Friggens 1990)

s: Heat production from carbohydrate conversion to fat; 3.12 (Friggens 1990) kJ/g CHO

t: Supply of endogenous protein.

u, v, w: Energy content of protein, fat and carbohydrate (Appendix 1, Table iii.)

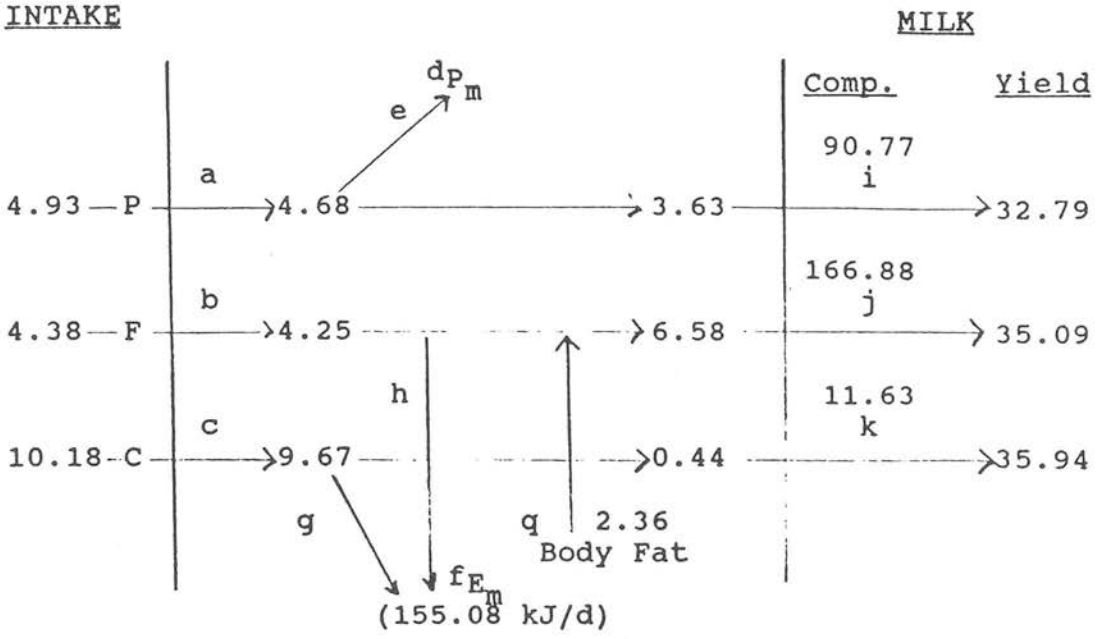
Body Protein: The quantity used for the estimation of maintenance requirments were; For dams offered the 215 gCP/kg DM feed that of group HH experiment E1, while for the other females values were derived from experiment E3.

Milk Composition: For females offered the 215 and 90 gCP/kg feed, milk composition were those reported in experiment E4, while the milk composition of moderately restricted dams was assumed to be equivalent to that of the high protein group.

Maintenance Energy Requirments: These are assumed to be met primarily by oxidising carbohydrate and if energy requirements exceed that available from carbohydrate then fat is oxidised.

Endogenous Nutrients: Assumed to be used for milk production with the same efficiency as dietary nutrients.

Surplus Nutrients: Assumed to be completly oxidised.



Milk Yield = 32.79 g/d	Heat Production
2.97 P	49.60 l
5.47 F	24.07 m
0.38 C	0.34 n
	74.01 kJ

Surplus Nutrients	Heat Production
0.43 g F	17.03 u
0.04 g C	0.67 v
	17.70 kJ

Total Heat Production

155.08
74.01
17.70
246.79 kJ/d

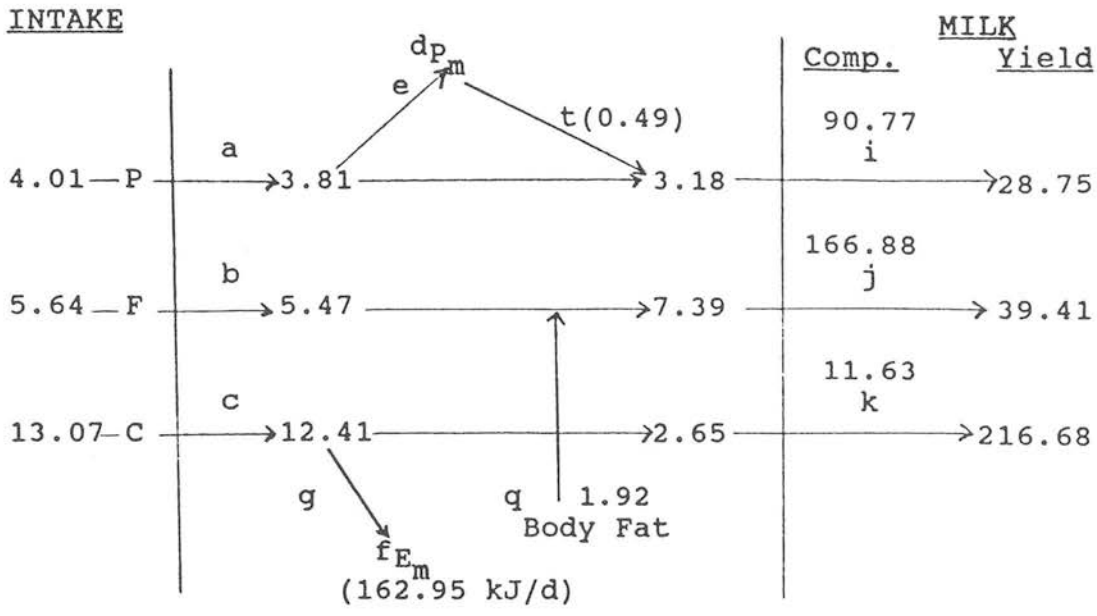
Heat Capacity = 393.83 kJ/d

Litter Growth

Protein Requirements:
Maintenance^{d} = 0.24
Gain = 1.88
2.12 g/d = 2.62^{ae} g/d

P Protein, C Carbohydrate, F Fat.
Intake, g/d; Composition, mg/g; Yield, g/d.

Fig. 7.2. Nutrient balance for females offered the 215 gCP/kg DM diet on day 3 of lactation.



Milk Yield = 28.75 g/d
2.61 P
4.80 F
0.33 C

Heat Production
43.59 l
21.12 m
0.30 n
65.01 kJ

Surplus Nutrients
1.95 g F
2.30 g C

Heat Production
77.22 u
38.41 v
115.63 kJ

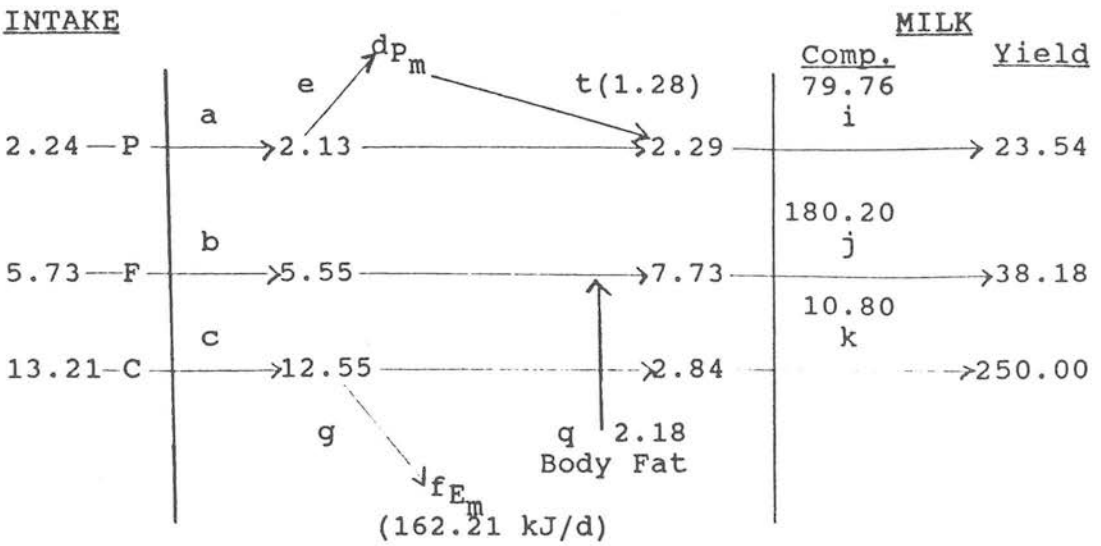
Total Heat Production
162.95
115.63
65.01
343.59 kJ/d

Heat Capacity = 389.81 kJ/d

Litter Growth
Protein Requirements:
Maintenance[d] = 0.24
Gain = 2.16
2.40 g/d = 2.96^{ae} g/d

P Protein, C Carbohydrate, F Fat.
Intake g/d; Composition, mg/g; Yield, g/d.

Fig. 7.3. Nutrient balance for females offered the 150 gCP/kg DM diet on day 3 of lactation.



Yield (without endogenous protein) = 10.41 g/d, Heat Production 452.90 kJ/d.

Yield (t = 1.28 g/d) = 23.54 g/d		Heat Production	
1.88 P		31.40	l
4.24 F		18.66	m
0.25 C		0.22	n
		50.28	kJ

Surplus Nutrients	Heat Production	
2.97 g F	117.61	u
2.58 g C	43.09	v
	160.67	kJ

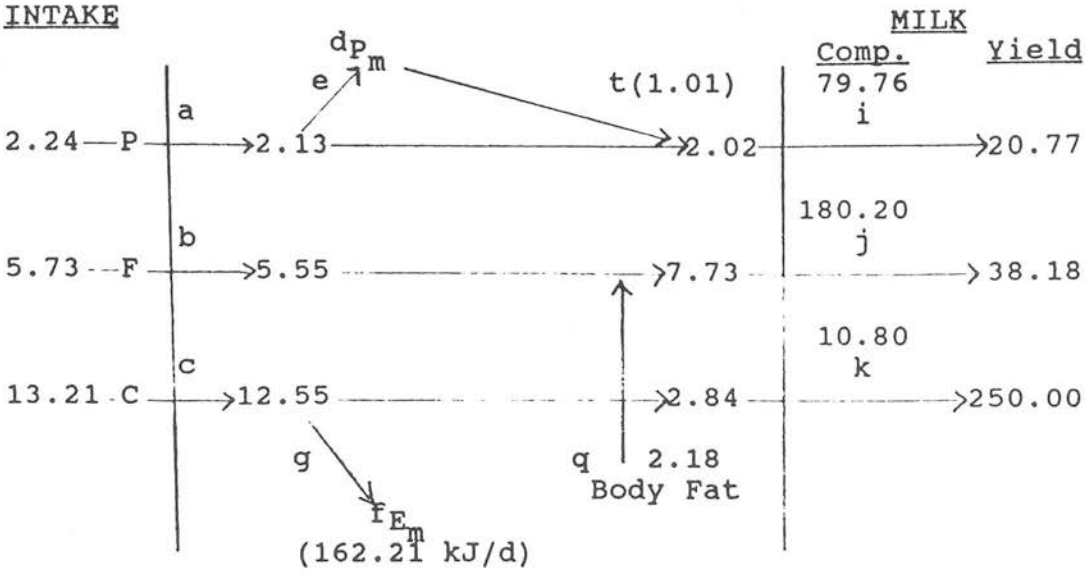
Total Heat Production
162.21
160.67
50.28
373.16 kJ/d

Heat Capacity = 372.63 kJ/d

Litter Growth
Protein Requirements:
Maintenance^{d} = 0.22
Gain = 1.51
1.73 g/d = 2.13^{ae} g/d

P Protein, F Fat, C Carbohydrate
Intake, g/d; Composition, mg/g; Yield, g/d.

Fig. 7.4a. Nutrient balance for females offered the 90 gCP/kg DM diet on day 3 of lactation.



Yield (t = 1.01 g/d) = 20.77 g/d
= 1.66 P
= 3.74 F
= 0.22 C

Heat Production
27.22 l
16.46 m
0.20 n
44.38 kJ

Surplus Nutrients
3.53 g F
2.61 g C

Heat Production
139.79 u
43.59 v
184.38 kJ

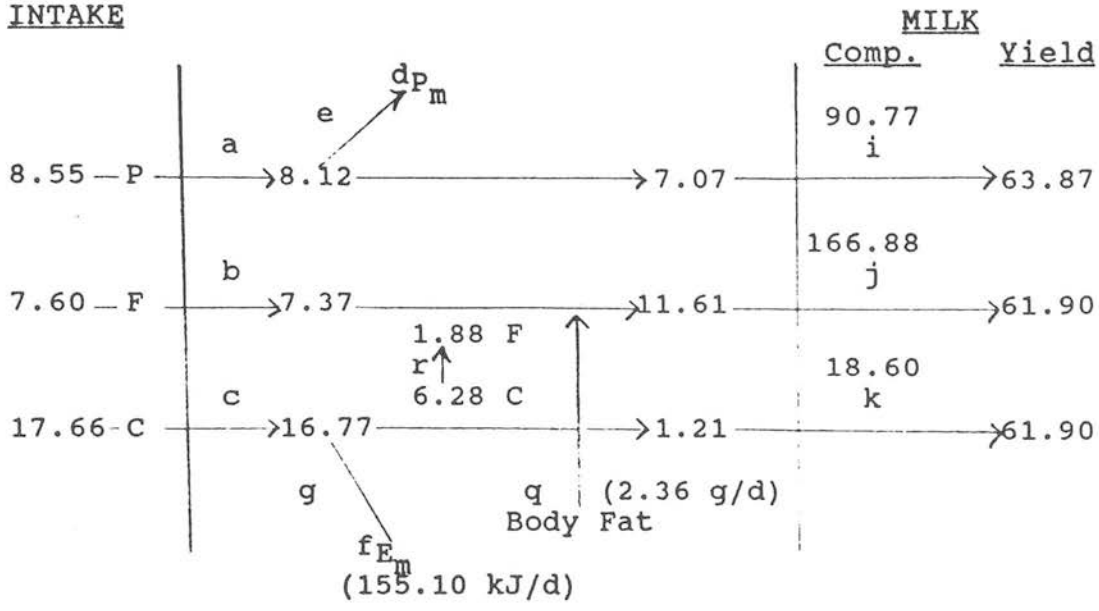
Total Heat Production
162.21
184.38
44.38
390.97 kJ/d

Heat Capacity = 372.63 kJ/d

Litter Growth
Protein Requirements:
Maintenance^{d} = 0.22
Gain = 1.51
1.73 g/d = 2.13^{ae} g/d

P Protein, F Fat, C Carbohydrate.
Intake, g/d; Composition, mg/g, Yield, g/d.

Fig.7.4b. Nutrient balance for females offered the 90 gCP/kg DM diet on day 3 of lactation.



Milk Yield = 61.90 g/d
5.62 P
10.33 F
1.15 C

Heat Production
93.85 l
45.45 m
1.03 n
140.33 kJ

Surplus Nutrients
0.22 g P

Heat Production
5.19 kJ w

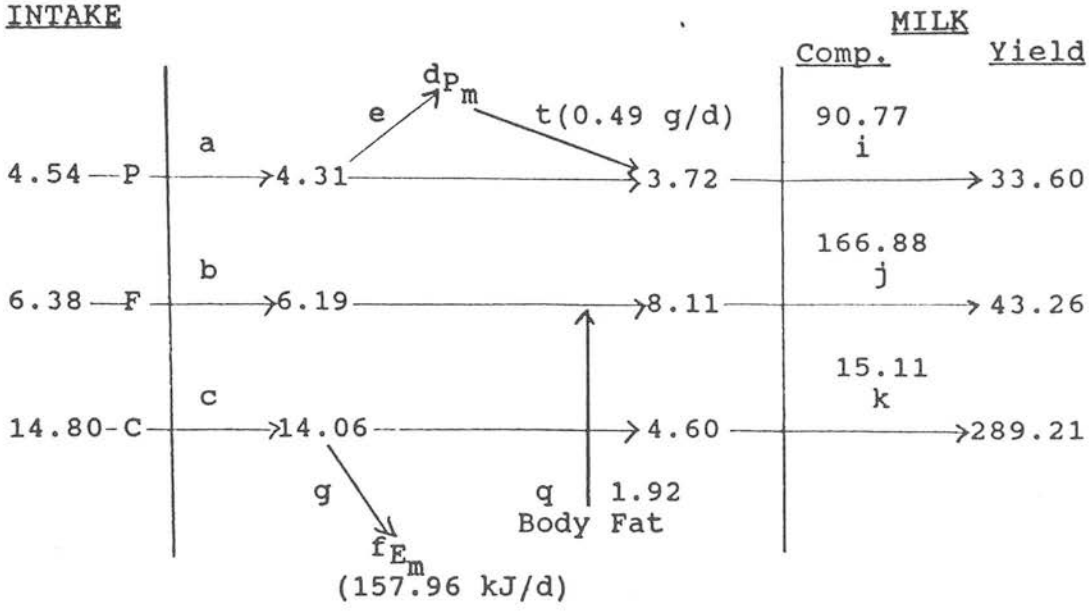
Total Heat Production
155.10
140.33
19.59 s
5.19
320.21 kJ/d

Heat Capacity = 393.83 kJ/d

Litter Growth
Protein Requirements:
Maintenance^{d} = 0.61
Gain = 4.11
4.72 g/d = 5.84^{ae} g/d

P Protein, F Fat, C Carbohydrate.
Intake, g/d; Composition, mg/g; Yield, g/d.

Fig. 7.5. Nutrient balance for females offered the 215 gCP/kg DM diet on day 9 of lactation.



Milk Yield	= 33.60 g/d	Heat Production	
	= 3.05 P	50.93	l
	= 5.61 F	24.68	m
	= 0.51 C	0.46	n
		76.07	kJ

Surplus Nutrients	Heat Production	
1.81 g F	71.68	u
4.06 g C	67.80	v
	139.48	kJ

Total Heat Production

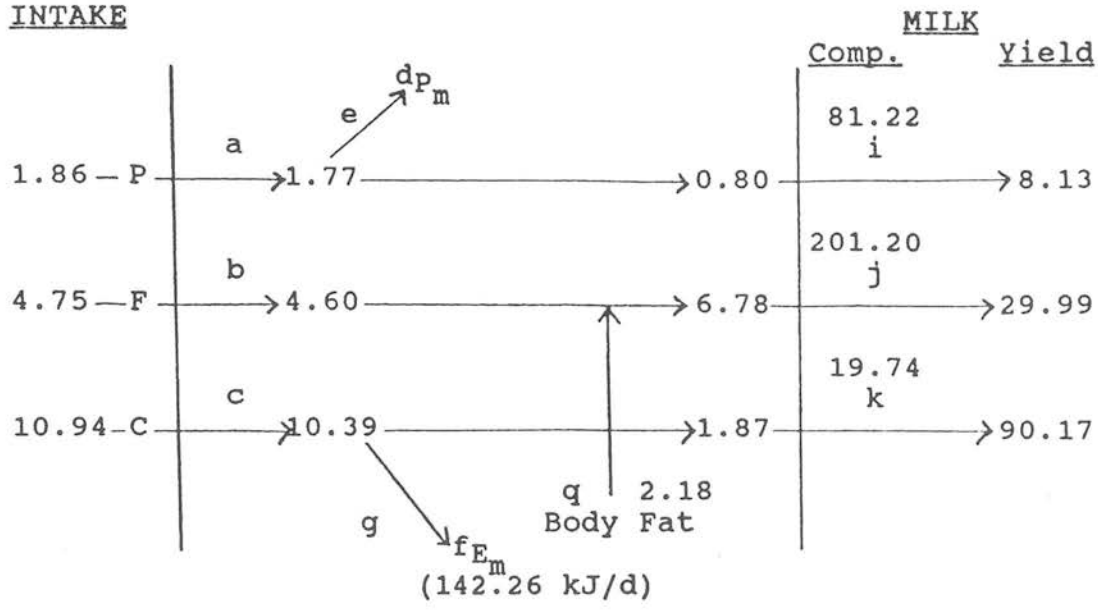
157.96
139.48
76.07
373.51 kJ/d

Heat Capacity = 380.79 kJ/d

Litter Growth
Protein Requirements:
Maintenance^{d} = 0.38
Gain = 2.41
2.79 g/d = 3.45^{ae} g/d

P Protein, F Fat, C Carbohydrate.
Intake, g/d; Composition, mg/g; Yield, g/d.

Fig. 7.6. Nutrient balance for females offered the 150 gCP/kg DM diet on day 6 of lactation.



Milk Yield =		Heat Production	
	8.13 g/d		
	0.66 P	11.02	l
	1.64 F	7.22	m
	0.16 C	0.14	n
		18.38 kJ	

Surplus Nutrients		Heat Production	
	4.94 g F	195.62	u
	1.70 g C	28.39	v
		224.01 kJ	

Total Heat Production

142.26
224.01
18.38
384.65 kJ/d

-86.33 (Body Fat)
298.32 kJ/d

Heat Capacity = 318.44 kJ/d

Litter Growth
Protein Requirements:
Maintenance^{d} = 0.40
Gain = 0.50
0.90 g/d = 1.11^{ae} g/d

P Protein, F Fat, C Carbohydrate
Intake, g/d; Composition, mg/g; Yield, g/d.

Fig. 7.7. Nutrient balance for females offered the 90 gCP/kg DM diet on day 11 of lactation.

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APPENDIX 1

INTRODUCTION

The four experiments (E1, E2, E3 and E4) described in the previous chapters (2 - 6) are reported in chronological order. In this appendix descriptive statements not followed by an experiment number refer to all experiments. Since the data described in Chapters 5 and 6 are derived from one experiment (E4), general statements concerning E4 applies to both chapters. All four experiments have a common general methodology concerning animals, environmental conditions and diet formulations, while more precise information on experimental protocol is given in the relevant chapters.

SYNOPSIS

Female Sprague-Dawley rats were caged individually and offered experimental feeds and water *ad libitum*. Females were subjected to various dietary treatments during gestation and lactation, and were allowed to nurse a standardised litter until either day 13 (E1 and E2) or 12 (E3 and E4) of lactation. During the experimental period maternal feed intake, weight change and litter live weight gain were recorded daily. Changes in maternal carcass composition were measured during gestation and lactation, while changes in tissue protein metabolism, energy expenditure and milk composition were measured throughout lactation.

ANIMALS

In all experiments pathogen free Sprague-Dawley rats (Harlan Olac UK Ltd.) were used. All females were to have already produced one litter and in all experiments experienced males were used for mating. Before the start of each experiment, all animals were allowed to adjust to their new environment for seven days. At the appropriate time females were placed in a wire bottomed cage with a proven male for mating. The morning on which mating was confirmed, through the presence of vaginal plugs, was designated day 1 of gestation and the females were returned to solid bottomed plastic cages for the remainder of the experiment.

Following this another female was placed in with the male, and this process continued until all females had apparently conceived.

Table i. General animal numbers, conception rates and litter size from the four rat experiments.

Experiment Number	E1	E2	E3	E4
Female Number	40	48	66	80
Male Number	20	25	20	20
Conception Rate	0.92	0.90	0.88	0.74
Health Problems %	0	0	0	10
Useful Females %	92.5	87.5	83.3	40.0
Mean Litter Size	13.7	12.4	12.8	8.7
Pup Mortality %	2	3	5	7

For each experiment the female/male numbers, conception rate and mean litter size are shown in Table i. For experiments E1, E2 and E3 conception rates and litter size were high enough to ensure adequate numbers of females for each study. However, it can be seen that in E4 only 40 % of the females supplied were able to be used. This dramatic reduction in available female numbers resulted from a combination of conception difficulties, mammary tumours and a considerably lower litter size. As a result of this, the original objectives of experiment 4 were abandoned and only the milk composition and tissue protein synthesis of females offered high and low protein diets during lactation were measured. The suppliers were unable to provide a satisfactory explanation for such a severe reduction in female numbers, although it is possible, from the proportion of females with mammary tumours, that some of the females supplied were considerably older than requested.

Such studies involving lactating rats would be considerably easier if the females could be drawn from a larger breeding population where difficulties associated with the conception rate and natural litter size could be overcome even when standardising litters to 12 pups.

ENVIRONMENTAL CONDITIONS

All females were caged individually in solid bottomed plastic cages (45cm x 28cm x 20cm) with sawdust for bedding (Gold Chips; B.S. & S, Edinburgh). All experiments had 12 hour light/dark photoperiods, with the light period in experiments E1, E2 and E3 from 0800 - 2000 hours and from 1100 - 2300 hours in experiment E4. A radio-timer was also pre-set to come on with the lights and entry to this room was not effected until after this time.

The animal rooms were all maintained at 22 °C and with relative humidity from 40 - 60 %. The room temperatures for each experiment are shown in Table ii. Experiment E1, unlike E2, E3 and E4, was carried out in a different animal house and because of a short period of extremely hot weather, temperatures approached maximum recorded values of 27 °C. No such problems were encountered in the other experiments.

Table ii. Animal room temperature (°C) for the four rat experiments.

Experiment	E1	E2	E3	E4
Mean Temp. (°C)	21.8	21.6	22.8	21.5
SD	1.5	1.0	0.9	1.2
Max	26.8	24.2	25.2	24.5
Min	20.0	20.5	21.2	21.5

FEMALE HANDLING and CROSS FOSTERING

Throughout gestation, dams were handled and weighed daily in an attempt to limit the impact of such disturbances during lactation on lactational performance and pup mortality. Since female rats with litters are extremely nervous, they were always approached first and allowed a period of recognition before the litter was disturbed.

For all females, the day of parturition was designated day 1 of lactation and litters were adjusted to a size of 12 pups. The natural variation in litter-size necessitated the cross-fostering of pups between litters. In experiment E4 the lower litter-size (Table i) and the spread of births resulted in the loss of a number of females from the experiment. Cross-fostering was accomplished by gently rubbing the fostered pups with the bedding and pups of the recipient dam, which was temporarily in a separate cage, and then placing them with the

natural litter. In experiments E1 and E2, when dietary protein restriction was imposed during gestation, an attempt was made to standardise both litter size and weight, although this was not always possible. Dams that could not receive a standardised litter from day 1 of lactation were removed from the experiment.

FEED INGREDIENTS, FORMULATION and MANUFACTURE

The three major feed components are protein, carbohydrate (CHO) and fat. In all experiments, dietary protein was provided by casein (Scottish Milk Marketing Board) supplemented with DL-Methionine (0.99/0.01) and CHO was supplied by a 2:1 mixture of corn starch and sucrose. In experiments E1, E3 and E4 the dietary fat source was corn oil while in experiment E2 solid vegetable fat was used. The main components were supplemented with vitamins and minerals in quantities that meet the requirements of a lactating rat (NRC 1978).

The objective of the four experiments was to investigate the effects of variations in dietary protein content, but not energy, on various aspects of nutrient partitioning during lactation. It was therefore essential that all diets used were formulated to be isoenergetic i.e. both their protein content and protein:energy ratio varied. For this to be achieved, the energy lost following a reduction in dietary protein content was replaced by increasing the dietary CHO and fat contents in a fixed ratio that was dependent upon their gross energy content. The equation used to calculate the proportions of CHO and fat required to replace dietary protein is shown below:

$$\text{Proportion of CHO} = \frac{(GE_{\text{Protein}} - GE_{\text{Fat}})}{(GE_{\text{CHO}} - GE_{\text{Fat}})}$$

Proportion of Fat = 1 - (Proportion of CHO)

GE:- (KJ/g DM)

Once the proportions of protein, CHO and fat have been calculated, fixed amounts of vitamin and mineral supplements were added to complete the final diet formulation (100%).

In doing this the isoenergetic nature of the diets was maintained, although at a slightly lower energy density. All diets were formulated on a dry matter basis and the actual diet composition and chemical analysis for each experiment are given in the relevant chapters. The gross energy contents of the protein, CHO and fat used in each experiment are shown in Table iii.

Table iii. The gross energy content (kJ/g DM) of the major feed ingredients used in the four rat experiments.

Experiment	E1	E2	E3	E4
Protein[a]	23.61	23.28	23.63	23.63
CHO[b]	16.71	16.30	16.71	16.71
FAT	39.65	38.91[c]	39.62	39.62

[a] Casein (fat free) + DL Methionine (0.99 + 0.01)
[b] Carbohydrate: Starch/sucrose (2:1)
[c] Solid vegetable fat

In order for accurate measurements of feed intake to be made, it was necessary to create a feed that was too soft for females to be able to remove lumps from the feed container, but firm enough to prevent losses if the feed container was knocked over. This was achieved by adding water to all feeds, with the amount required influenced by the diet's oil content. As a result of this all feeds were stored frozen (-20 °C). However, despite this added water, changes in the dry matter content of feed samples kept at room temperature (22 °C) for 24 hours were negligible. Due to the physical nature of the diets all females were given a plastic chew ring to allow the natural trimming of teeth. The dry matter content of all diets used in each experiment is shown in Table iv.

Table iv. The dry matter content (%) of the diets used in the four rat experiments.

Experiment Dietary Protein (%)	E1	E2	E3	E4
21.5	71.52	67.79	70.35	68.50
15.0	-	-	81.04	-
9.0	79.51	79.71	83.81	81.96
6.5	84.69	82.41	-	-

In order to maintain the dietary energy density, the diets used in experiments E1, E3 and E4 are associated with high levels of corn oil. If untreated, this oil would separate out and so to prevent this an emulsifier (lecithin) was included in all diets (E1, E3 and E4) at 0.2 % (fresh weight). Such problems were not encountered with diets from experiment E2. All diets were also supplemented with an antioxidant (butylated hydroxy toluene) at 0.001% (fresh weight) to limit lipid oxidation.

The vitamin and mineral supplements (S.D.S. Edinburgh) used in experiments E1 and E2 were included in all feeds at 50 and 100 g/kg DM respectively. However, for experiments E3 and E4 an alternative source of vitamin and minerals were used (ICN Nutritional Biochemicals, USA) and they were both included in feeds at 50 g/kg DM. Since corn starch was used as a filler in the supplements supplied by S.D.S, all diets used in experiments E3 and E4 were balanced by it's addition at 43 g/kg DM. The ICN vitamin and mineral mixtures also lacked a source of choline and cobalt, therefore all feeds were also supplemented with choline chloride (6.7 g/kg DM) and vitamin B_{12} (40 μg/kg). The composition of the vitamin and mineral mixtures used in experiments E1/E2 and E3/E4 are shown in Tables v and vi/vii.

All feeds for each experiment were produced in one batch using a commercial mixer and then stored frozen in sealed plastic containers each holding approximately 1.5 kg.

DIET FEEDING, FEED INTAKE and COMPOSITION

The synthetic diets, described above, were introduced to all females from day 1 of gestation. Weighed samples of feed were offered in pre-weighed 120 ml Beatson jars, which were held upright in the cage, to prevent losses, by plastic jar holders. Fresh feed was made available every morning and enough was provided to ensure that feeding was *ad libitum*. Daily feed intake was recorded as the difference between the weight of feed provided and refused. The experimental period was ended on day 13 of lactation because from day 14 onwards feed intake measurements can become less reliable as pups begin to open their eyes

and therefore have access to solid food. In all experiments feed samples were taken from each batch used and stored collectively for compositional analysis. Feed dry matters were estimated by drying duplicate samples at 60 °C for 48 hours. Feed samples were also analysed for protein and gross energy by techniques used for carcass analysis (see later). For each experiment, the results of this analysis are shown in the relevant chapters.

Table v. Composition of the vitamin and mineral supplements used in experiments E1 and E2.

VITAMIN		MINERAL	
	mg/kg		g/kg
Pantothenate	250	Calcium	63.7
Niacin	4947	Phosphorus	101.6
B_6	490	Sodium	38.2
B_{12}	1.2	Potassium	36.0
K_3	50	Magnesium	25.2
B_2	750	Sulphur	3.7
Folate	990	Chloride	59.0
Choline	25000	Iron	0.7
B_1	300	Manganese	0.7
Biotin	9.4		
	i.u./g		mg/kg
A	800	Zinc	150.3
D_3	100	Selenium	1.2
E	6	Copper	50.8
		Cobalt	10.0
		Iodine	35.8

The filler used in both supplements was corn starch
For inclusion rates see relevant chapters

Table vi. The composition (mg/kg) of the Vitamin Mix (ICN Nutritional Biochemicals) used in experiments E3 and E4.

Vitamin	mg/kg
Thiamine HCl (B_1)	600
Riboflavin (B_2)	600
Pyridoxine HCl (B_6)	700
Nicotinic Acid (Niacin)	3000
D-Calcium Pantothenate	1600
Folic Acid	200
D-Biotin	20
Cyanocobalamin (B_{12})	1
Retinyl Palmitate (A) 250 i.u/mg	1600
dl-Tocopherol Acetate (E) 250 i.u/g	20000
Cholecalciferol (D_3) 400 i.u/mg	2.5
Menaquinone (K)	50

Filler used was finely powdered sucrose

Table vii. The composition (g/kg) of the Mineral Mix (ICN Nutritional Biochemicals) used in experiment 3 and 4.

Mineral	g/kg
Calcium Phosphate, Dibasic ($CaHPO_4$)	500
Sodium Chloride (NaCl)	74
Potassium Citrate, Monohydrate	220
Potassium Sulphate (K_2SO_4)	52
Magnesium Oxide (MgO)	24
Manganous Carbonate (43 - 48 % Mn)	3.5
Ferric Citrate (16 - 17% Fe)	6
Zinc Carbonate (70% ZnO)	1.6
Copper Carbonate (53 - 55% Cu)	0.3
Potassium Iodate (KIO_3)	0.01
Sodium Selenite ($Na_2SeO_3.5H_2O$)	0.01
Chromium Potassium Sulphate	0.55

Filler was finely powdered sucrose

MEASUREMENTS MADE DAILY

During the experimental period (gestation and lactation) maternal body weight, feed intake and litter weight changes were recorded daily. Dam and litter live-weights were measured using a Satorious animal balance which estimated the weight as the mean of 10 automatic readings. In experiment E3 the distribution of dam feed intake and litter weight gain between the two photoperiods were estimated by recording the feed intake and litter weight at the start and end of each 12 hour period.

CARCASS and TISSUE ANALYSIS

At the appropriate time during each experiment, groups of females were used in the analysis of tissue metabolism (E2, E4), milk composition (E4) or slaughtered for subsequent carcass analysis (E1, E3). In all experiments dams were culled by decapitation using a hand operated guillotine. Following slaughter, the bodies of females used for carcass analysis were dissected and the organs and adipose stores of the abdominal and thoracic cavities were removed and weighed. The liver, mammary gland (total) and gastrointestinal tracts were all kept for tissue analysis. The gut contents were emptied through incisions made in the stomach, small and large intestines followed by gentle squeezing of the digesta. Empty guts were then rinsed with saline, blotted dry and trimmed of excess fat. Tails were also removed

from all bodies. Following dissection, the empty carcass and major organs were quickly frozen for storage. The procedures used following the slaughter of dams in experiments E2 and E4 will be discussed later. All litters were also slaughtered by decapitation and their bodies were quickly frozen without dissection. In the subsequent analysis, the carcass, organs and litters were all treated in a similar fashion.

Dry Matter

The dry matter content of a frozen carcass was estimated by freeze drying it to constant weight in an Edwards Purini 501 freeze dryer. The dried empty carcass was minced and then milled in a Retsch ultra-centrifugal mill that was designed to prevent overheating of samples and thus the consequent loss of fat. The dried organs were milled in a water-cooled tissue mill. The dried ground samples were all re-frozen and then freeze dried for a further 24 hours to remove any water remaining or picked up in the milling procedure. Following this, all dried samples were stored with dessicant prior to analysis. All subsequent analyses were carried out on duplicate samples.

Tissue Nitrogen and Crude Protein

Dried tissue samples were analysed for their nitrogen/protein contents using the micro-kjeldahl technique. An accurately weighed tissue sample (0.25 g) was placed into a kjeldahl flask and digested with 5 ml concentrated H_2SO_4, a reaction catalyst (75 % K_2SO_4, 25 % $CuSO_4$) and anti-bumping granules. During the digestion, the organic nitrogen was converted to ammonium sulphate. Following digestion the contents of the kjeldahl flask were washed into a 100 ml volumetric flask with distilled water and made up to volume. A 5 ml aliquot of this solution was then added to a Hoskins distillation unit along with 5 ml 5 M NaOH and 10 ml distilled water. The ammonia nitrogen in the digestion mixture was then liberated by steam distillation and collected by 0.16 M H_3BO_3 in a conical flask. The quantity of liberated nitrogen in the conical flask was then determined by titrating it against a

known molarity of HCl (M/140). The quantity of nitrogen and therefore protein in the original dried sample can be estimated using the following calculation:

Assume 1 ml M/140 HCl* = 0.1 mg N.

* For standardisation of acid see below.

Titre x 0.1 = mg N in conical flask

(Titre x 0.1) x 20 = mg N in volumetric flask

$$\frac{(\text{Titre} \times 0.1) \times (20)}{1000} = \text{g N in sample}$$

$$\frac{(\text{Titre} \times 0.1) \times (20)}{1000} \times 6.25 = \text{g protein in sample :- A}$$

Sample Protein (g/g DM) = A / sample weight

Standardisation of Acid:- In the above calculation 1 ml of M/140 (0.007142 M) HCl was assumed to be equivalent to 0.1 mg nitrogen. However, if the nitrogen/protein content of a sample is to be determined accurately, the actual molarity of HCl used needs to be estimated. This is achieved by standardising the HCl against a solution of known nitrogen content. A 5 ml aliquot of 0.00357 M $(NH_4)_2SO_4$ is distilled, as above, and the resultant ammonium nitrogen is titrated against the unknown HCl.

5 ml 0.00357 M $(NH_4)_2SO_4$ = (A) ml of (B) M HCl

$$\text{(B) M HCl} = \frac{5 \times 0.00357}{(A)} \times 2^*$$

if 1 ml 0.007142 M HCl = 0.1 mg Nitrogen

1 ml (B) M HCl = (E) mg N

$$= \frac{0.1 \times (B)}{0.007142}$$

1 ml (B) M HCl = (F) mg N

* 1 mole M/280 $(NH_4)_2SO_4$ = 2 moles M/140 HCl

Following this standardisation, the quantity of nitrogen (F) can be used in the calculation of sample nitrogen content.

Carcass Gross Energy and Fat

The gross energy content was estimated using a Gallenkamp adiabatic bomb calorimeter and involves the complete combustion of a known weight of sample in a steel bomb containing an oxygen rich atmosphere. The energy liberated is determined from the temperature rise, total heat capacity and the following calculation:

$$\text{Gross Energy KJ/g DM} = \frac{((T \times HC) - C)}{\text{sample weight}}$$

where; T:- temperature rise (°C)
HC:- heat capacity (J/°C)
C:- correction factor for heat gain (J)

Since the energy liberated from dried carcass samples is derived totally from the combustion of organic matter, of which body fat and protein can be assumed to provide the major contribution, and that this measured value agrees closely with the energy content calculated from the contributions made by it's constituents (Friggens 1990), the carcass fat content in this study was calculated using the following equation:

Carcass Fat (g/g DM) = (GE - (23.6 x crude protein))/39.6

where; GE:- carcass gross energy (kJ/g DM)
Crude Protein:- carcass protein content (g/g DM)
23.6*:- gross energy content of protein (kJ/g)
39.6*:- gross energy content of fat (kJ/g)

* McDonald et al. (1981)

Carcass Ash

The carcass ash content was estimated by heating dried samples in a muffle furnace at 550 °C for 24 hours.

In the above analyses, if the results of duplicate samples varied by more than 5 % further replicates were analysed. The dry matter content of the dried samples was checked

throughout the analytical procedure by heating samples at 60 °C, although few discrepancies were found.

TISSUE PROTEIN SYNTHESIS

In experiments E2 and E4 (Chapter 6) rates of tissue protein synthesis were measured *in vivo* using the flooding dose technique of Garlick *et al.* (1980). Rates of protein synthesis were estimated at various stages during lactation in the liver, mammary gland, duodenal mucosa and skeletal muscle (gastrocnemius) from the incorporation of [^{3}H] phenylalanine into tissue protein.

While being restrained by hand in a cloth, dams were injected via a lateral tail vein, using a 1/2 inch 26G needle, with a solution containing 150 mM L-phenylalanine and 50 μCi/ml L-[2,6 ^{3}H] phenylalanine at a rate of 1.0 ml/100 g body weight and then returned to their litter. After 10 minutes dams were decapitated and the tissue samples required were quickly excised and plunged into liquid nitrogen. Tissue samples were then stored frozen for subsequent analysis. In this study no correction was made for the gradual linear decline of tissue free phenylalanine specific activity during the 10 minute incorporation period, since previous studies have shown that the rate of decline in mammary tissue, liver and muscle is slow and insignificant (Garlick *et al.* 1983, Sampson *et al.* 1986). In the calculation of synthetic rate, the actual incorporation time used takes account of the additional period between slaughter and tissue cooling. The separation of duodenal mucosa and serosa is described in the relevant chapter.

The incorporation of [^{3}H] phenylalanine into tissue protein, and thus the rate of protein synthesis, was assayed in triplicate using the procedure described in Fig. i. After a frozen tissue sample had been first powdered by pulverising it between two metal blocks cooled in liquid nitrogen, the free and protein bound phenylalanine were separated. Following the hydrolysis of tissue protein, both the free and bound phenylalanine were then converted to a derivative (ß-phenethylamine) and their specific activities were estimated. This procedure is

a slightly modified version of that described by Garlick *et al.* (1980) and was obtained from a personal communication with P.J. Garlick. The conversion of phenylalanine to ß-Phenethylamine required the activity of L-tyrosine decarboxylase (Sigma Chemical Co.). This enzyme was suspended (0.7 and 1.4 unit/ml for supernatant and hydrolysate respectively) in 0.5 M sodium citrate (pH 6.3) containing the co-factor pyridoxal phosphate (0.5 mg/ml).

Following removal of the organic layer (Fig. i), the frozen aqueous layer (0.01 M H_2SO_4) was used for counting and ß-Phenethylamine estimation. A 1 ml supernatant and 2 ml hydrolysate sample (3 ml muscle hydrolysate) were counted with a liquid scintillation cocktail in a Beckman LS 5000CE scintillation counter. A further 1 ml of supernatant or 0.05 ml hydrolysate were assayed for ß-Phenethylamine. To 1.0 ml of the ß-Phenethylamine solution was added 0.5 ml of 2 mM L-leucylalanine, 1.0 ml of 50 mM ninhydrin and 2.5 ml of 1 M potassium phosphate, pH 8.0. Samples were incubated at 60 °C for 1 hour and then cooled in ice for 15 minutes. The fluorescence of this solution at 495 nm (excitation 390 nm) was measured in a Perkin Elmer LS30 fluorimeter, in which samples were retained in the flow cell for 5 s prior to reading. Results were calculated from comparison with similarly treated standards of 1 - 50 µM ß-Phenethylamine in 0.01 M H_2SO_4. All steps in this assay were carried out in the dark.

The fractional rate of protein synthesis (Ks) can be calculated from the following equation:

$$Ks = \frac{S_B \times 100}{S_A \times t}$$

where S_B and S_A represent the specific activities of protein bound and free phenylalanine respectively, and t is the time in days that elapsed between injection and the rapid cooling of tissue. An example of this calculation is shown in Fig. ii.

TISSUE RNA ANALYSIS

In experiments E2 and E4, tissue RNA contents were estimated using the method described by Munro *et al.* (1966, 1969). The assay used follows a similar procedure to that

of the protein synthesis assay previously described and from that assay supernatant C (Fig. i) contained the bulk of tissue RNA.

After the digestion of tissue protein (100 - 150 mg) using 10 ml 0.3 M NaOH (see section on protein synthesis assay), the protein and DNA were precipitated by adding 2 ml of 20 % $HClO_4$ and then cooling in ice. Following centrifugation, supernatant C was aspirated off and the precipitate was washed twice with 10 ml of 2 % $HClO_4$. These washings were combined with supernatant C and made up to a final volume of 100 ml and a perchloric acid concentration of 0.1 M. The ultraviolet absorption of this solution at 260 nm was then measured on a Beckman DU 65 spectrophotometer, zeroed using 0.1 M $HClO_4$. The calculation of RNA content involves the assumption that an extinction of 1.000 is equivalent to an RNA concentration of 32 μg/ml. For muscle samples the RNA content was estimated by measuring the UV absorption at both 260 and 232 nm, to correct for the possible presence of peptides, and then the equation developed by Ashford *et al.* (1986) shown below:

$$\text{RNA } (\mu g/ml) = 32.9_{A260} - 6.11_{A232}$$

TISSUE PROTEIN ANALYSIS

In a similar way to the RNA analysis, the protein synthesis assay provides a starting point for the estimation of tissue protein content. Following the digestion of tissue protein using 10 ml 0.3 M NaOH (see section on protein synthesis assay) (Fig. i), a 1 ml aliquot of this solution (B) was taken and it's protein content estimated using the method of Lowry *et al.* (1951). This assay involves the preparation of 2 solutions immediately before use:

1. Alkaline Copper Reagent

Solution A :- 50 mg $CuSO_4$ in 10 mls Na,K Tartrate (1 % w/v)

Solution B :- Add solution A to 100 mls 10% Na_2CO_3 in 0.5 M NaoH

2. Commercial Folin & Ciocalteu's phenol reagent diluted 1/10

The standard used was bovine serum albumin dissolved in 0.3 M NaOH at concentrations of between 25 - 200 μg/ml (Fig. iii). The assay procedure is as follows:

1. Add 1.0 ml of the alkaline copper reagent to 1.0 ml of unknown and standards. Mix and wait 10 minutes.

2. Add 3 mls of Folin reagent, mix and place in a water bath at 50 °C for 10 minutes.

3. After exactly 10 minutes, cool samples and measure extinction at 650 nm against a reagent blank.

The protein concentration of the unknown solution is then determined from the standard curve and the tissue protein content calculated by using the weight of the original sample.

TISSUE Na^+,K^+-ATPase (EC 3.6.1.3) ACTIVITY

In experiment E2, Na^+,K^+-ATPase activity, in the liver, mammary gland, muscle and duodenal mucosa, was measured *in vitro* through the inhibition of tissue oxygen consumption using Ouabain, a specific inhibitor of the Na^+,K^+-ATPase enzyme (Albers *et al.* 1968). Ouabain concentrations of 1 μM or greater have been shown to give maximal inhibition of Na^+,K^+-ATPase associated respiration (Gregg *et al.* 1982a).

Rates of tissue oxygen consumption were measured polarographically in a Rank oxygen electrode which contains a reaction chamber maintained at a constant temperature. At the base of this chamber are two electrodes immersed in a solution of saturated potassium chloride and separated from the rest of the chamber by a thin oxygen permeable teflon membrane. The rate of current flow between these two electrodes corresponds to the concentration of dissolved oxygen in the reaction medium and is recorded on a potentiometric chart recorder. Thus changes in oxygen concentration following tissue consumption will alter current flow and therefore the recorded output.

Before the oxygen electrode can be used, it must first be calibrated and the oxygen concentration of the reaction buffer determined.

Operational Check

Rinse reaction chamber with buffer

Fill reaction chamber with buffer

Add sodium dithionite to chamber (to remove all oxygen)

Record zero line

Electrode Calibration

Rinse reaction chamber with buffer*

Fill with fresh buffer (3 ml)

Record plateau on chart which represents the oxygen

concentration of the buffer

* Calibrated using air saturated KRB which has a known oxygen content (180 nmoles/ml).

Following calibration, weighed samples of liver snips, mammary gland slices (20 μm) (Bartley *et al.* 1976), muscle fibre bundles (tied with sutures) (Gregg *et al.* 1982b) and duodenal mucosal scrapes were washed and placed into reaction chambers containing 3 ml of Minimal Essential Medium (MEM) and 5 mM HEPES at 37 °C, pH 7.4. Tissue oxygen consumption was then recorded (pre-ouabain) for 10 minutes. After this time, ouabain was added to a final concentration of 10^{-4} M and the oxygen consumption was recorded for a further 10 minutes. The difference between the initial oxygen consumption and that following ouabain treatment was termed the Na^+,K^+-ATPase dependent respiration. The calculation of total, Na^+,K^+-ATPase dependent respiration and the percentage inhibition of the original oxygen consumption is shown in Fig. iv.

MAMMARY DNA ANALYSIS

In experiment E3, the mammary DNA content was assayed using the method of Munro *et al.* (1969). The assay procedure followed is described below.

A weighed sample of mammary tissue (150 mg) was homogenised with 5 ml of cold distilled water. To this homogenate, 2.5 ml of 0.6 M $HClO_4$ (ice cold) were added and allowed to stand for 10 mins. Following centrifugation (2800 x g, 15 min) the supernatant was discarded and the precipitate was washed twice with 5 ml of 0.2 M $HClO_4$. The precipitate was then dissolved in 4 ml 0.3 M KOH and heated at 37 °C for 1 hour. When solution was complete, a 0.5 ml aliquot was removed for subsequent protein analysis. To the remainder (3.5 ml), 2.5 ml of 1.2 M $HClO_4$ were added to precipitate the protein and DNA. After standing in ice for 10 min, the sample was centrifuged and the precipitate was washed twice with 5 ml of 0.2 M $HClO_4$. Following this the precipitate was re-dissolved in 5 ml of 0.3 M KOH and warmed until solution was complete. This solution was then made up to a final volume of 50 ml using 12 ml of 0.3 M KOH and distilled water. This solution contains the tissue DNA in 0.1 M KOH. A 2 ml sample was mixed with 1 ml of 0.04 % Indole and 1 ml concentrated HCl, heated in a boiling water bath for 10 min and then cooled under running water. This solution was extracted 3 times with 4 ml of $CHCl_3$, discarding the $CHCl_3$ phase. Prior to the last extraction the mixture was centrifuged at 500 rpm for 5 min. The extinction of the remaining aqueous layer was read at 490 nm.

The quantity of DNA present was obtained from a standard curve (Fig. v) prepared using standard DNA solutions that had also been subjected to the same procedure. The standards were produced by dissolving 20 mg calf thymus DNA (single strand) (Sigma Chemical Co.) in 50 ml distilled water and a small amount of NaOH. Standard DNA concentrations used were from 2 - 16 μg/ml. The calculation of tissue DNA content is shown in Fig. vi.

MILK COMPOSITION

Changes in the composition of rat milk during lactation and in response to dietary protein restriction were investigated in experiment E4. Milk samples were obtained from lactating rats on days 2, 4, 8 and 12 of lactation. In this study, the milking procedure was

designed to limit the impact of milk stasis (Grigor *et al.* 1986a), serial milking (Keen *et al.* 1980) and the diurnal variations in mammary lipogenesis and lactose synthesis (Williamson *et al.* 1984) on milk composition. Females were milked only once during lactation and they were initially separated from their litter for 2 hours at the start of the light period. During this period, dams had continual access to their allocated diet. Following this separation, dams were lightly anaesthetised (diethyl ether) and injected with 5 i.u. oxytocin (Sigma Chemical Co.). After a couple of minutes milk samples (0.5 - 0.75 ml) were obtained by gently stripping the left abdominal and thoracic teats. Milk samples were quickly cooled on ice and stored frozen (-20 °C) prior to subsequent analysis. During the development of this milking procedure, using additional females, various doses (0.1 - 5.0 i.u) of oxytocin were used. However, it was found difficult to obtain sufficient milk (> 0.5 ml) from severely protein restricted dams unless doses of > 4 i.u. oxytocin were used. In this study a dose of 5 i.u. was therefore used and despite the suggestion that this would alter milk composition (Linzell *et al.* 1975) (Na^+), the results described in experiment E4 do not reflect this. Although, this high dose of oxytocin may have precluded the accurate measurement of mammary protein synthesis (Chapter 6).

The milk samples collected were analysed for their macro and micro-nutrient content in the following way. All assays were carried out on sample duplicates.

Milk Lactose

Milk lactose content was assayed using a lactose test kit supplied by Boehringer Manneheim (Cat No. 176303). This assay involves the following conversions:

(1) Lactose + H_2O ----------> D-glucose + D-galactose
ß-Galactosidase

(2) D-Galactose + NAD^+ ----------> galactonic acid + NADH + H^+
Galactose dehydrogenase

The amount of NADH produced in reaction (2) is stoichiometric with the amount of lactose present. The increase in NADH is measured by means of its absorbance at 340 nm.

Prior to the assay, milk samples were first deproteinised using trichloroacetic acid (TCA). A weighed sample of milk (100 mg) was diluted with 1.0 ml distilled water and deproteinised with 50 µl of 3 M TCA. After standing at room temperature for 10 min, this solution was neutralised with 10 µl of 1 M NaOH and made up to 5 ml with distilled water. The protein was pelleted by spinning samples at 1300 x g for 30 s. Supernatant samples (50 µl) were then assayed for their lactose content.

The test kit consists of 4 reaction solutions used in the assay sequence shown below:

(1) Citrate buffer, pH 6.6, NAD, magnesium sulphate, stabilisers

(2) ß-galactosidase suspension (59 u/ml)

(3) Potassium diphosphate buffer, pH 8.6, stabilisers

(4) Galactose Dehydrogenase (0.9 u/ml)

	Blank Sample	Lactose Sample
Solution 1	0.20 ml	0.20 ml
Suspension 2	0.05 ml	0.05 ml
Sample	-------	0.05 ml
Mix and Incubate for 15 min at 20 - 25 °C		
Solution 3	1.00 ml	1.00 ml
Distilled water	2.00 ml	1.95 ml
Mix and Read Absorbance (A_1) at 340 nm after 2 min		
Suspension 4	0.05 ml	0.05 ml
Mix and Read Absorbance (A_2) at 340 nm after 20 min		

For both blank and lactose sample solutions the absorbance difference (A) was determined (A_2 - A_1). The absorbance difference of the blank was then subtracted from that of the lactose solution (A).

The lactose concentration of the reaction solution (3.3 ml) was then calculated using the following equation:

$$\text{Lactose (g / d)} = \frac{V \times MW \times A}{6{\cdot}3 \times d \times v \times 1000}$$

where; V = final volume (3.3 ml)
v = sample volume (0.05 ml)
MW = lactose molecular weight
d = light path (1 cm)
6.3 = absorption coefficient of NADH at 340 nm

The lactose concentration of the original milk sample was then calculated using the appropriate dilution factor and sample weight.

Milk Protein

Milk protein content was determined using the Coomassie Protein Assay Reagent (Pierce Chemical Co.). A weighed milk sample (20 mg) was diluted to 25 ml using distilled water. Subsequently, a 1 ml aliquot of this solution was mixed in a cuvette with 1 ml of the Protein Assay Reagent and, within 90 minutes, its absorbance was measured at 595 nm on a Beckman DU 65 spectrophotometer, zeroed with distilled water. Protein concentrations were then determined from a standard curve produced using casein (Sigma Chemical Co.) at concentrations of 0.25 - 2 mg/ml. Fresh standards were run with each assay. Sample blanks were also prepared and their absorbances subtracted from that of samples and standards. Milk protein concentrations were calculated using the dilution factor and sample weight.

Milk Lipid

The total milk lipid content was estimated using the extraction procedure developed by Bligh *et al.* (1959). A weighed milk sample (50 mg) was initially diluted with 2 ml of 0.9 % NaCl and then mixed with 4.4 ml of a chloroform:methanol (1:1 v/v) mixture. This combination ensured that the volumes of chloroform, methanol and water were in the ratio 2:2:1.8, which must be maintained if this procedure is to be used successfully. After being allowed to stand for 10 min, this mixture was centrifuged at 750 x g for 5 min to separate the

chloroform and alcoholic layers. The chloroform layer was then carefully removed and placed in a pre-weighed, dry test tube. A small volume of chloroform was left to ensure that none of the alcoholic layer was removed. The remaining alcoholic layer was then re-extracted twice with 2 ml of chloroform to remove any remaining lipid, with these extractions also added to the test tube. This test tube was then left overnight in a fume cupboard at 40 - 50 °C. The following morning, the test tube was re-weighed and the increase in weight taken as the total lipid content of the milk sample.

Milk Minerals

In this study, the major minerals investigated in the milk samples were sodium, potassium, calcium, phosphorus and magnesium. Milk samples (50 mg) were initially diluted to 2 ml with distilled water. This solution was then assayed for its mineral content using an Inductively Coupled Plasma Spectrometer (Jarell Ash ICAP 61E).

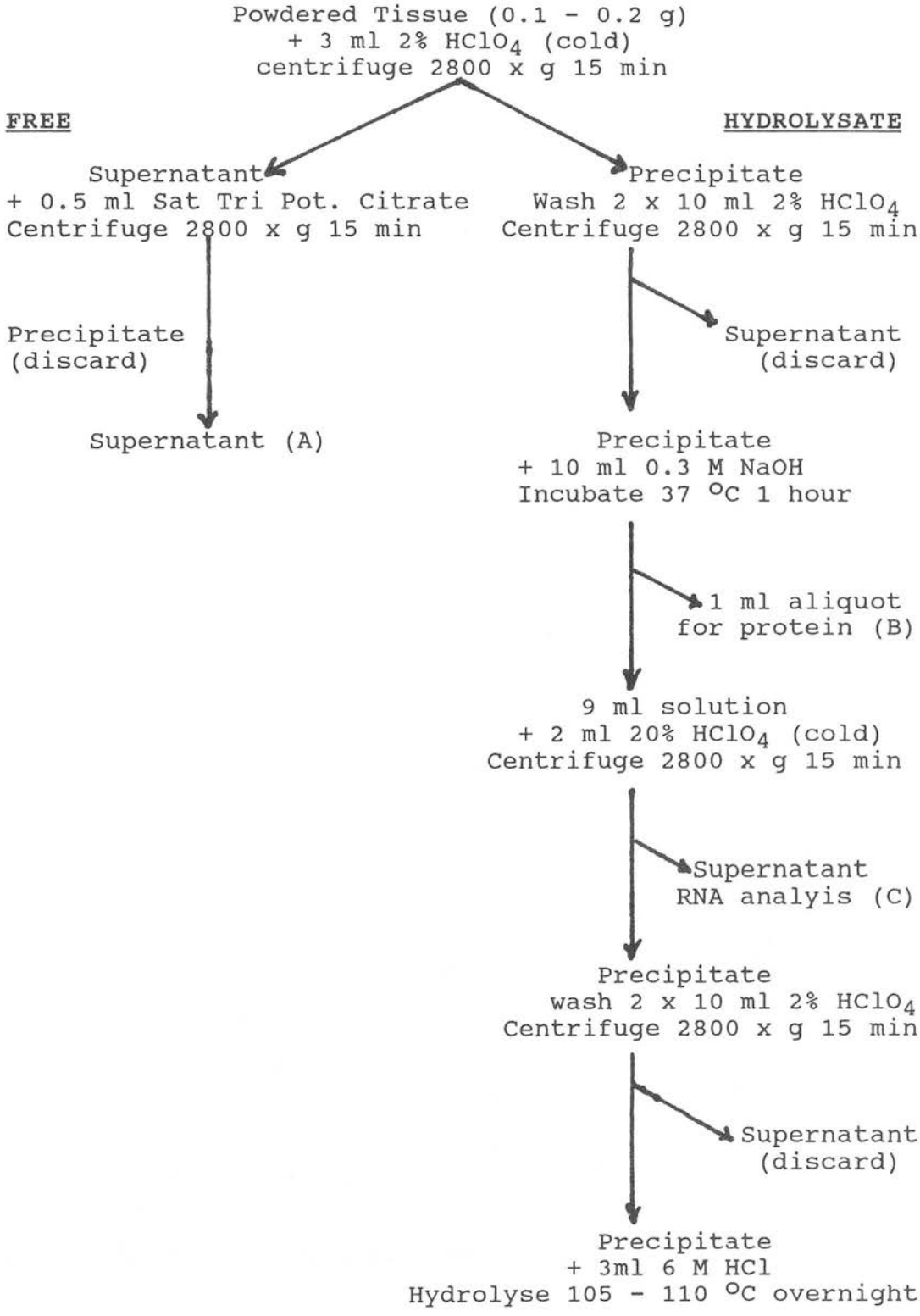

Fig.i. Assay procedure of Protein Synthesis Estimation
(Continued Overleaf)

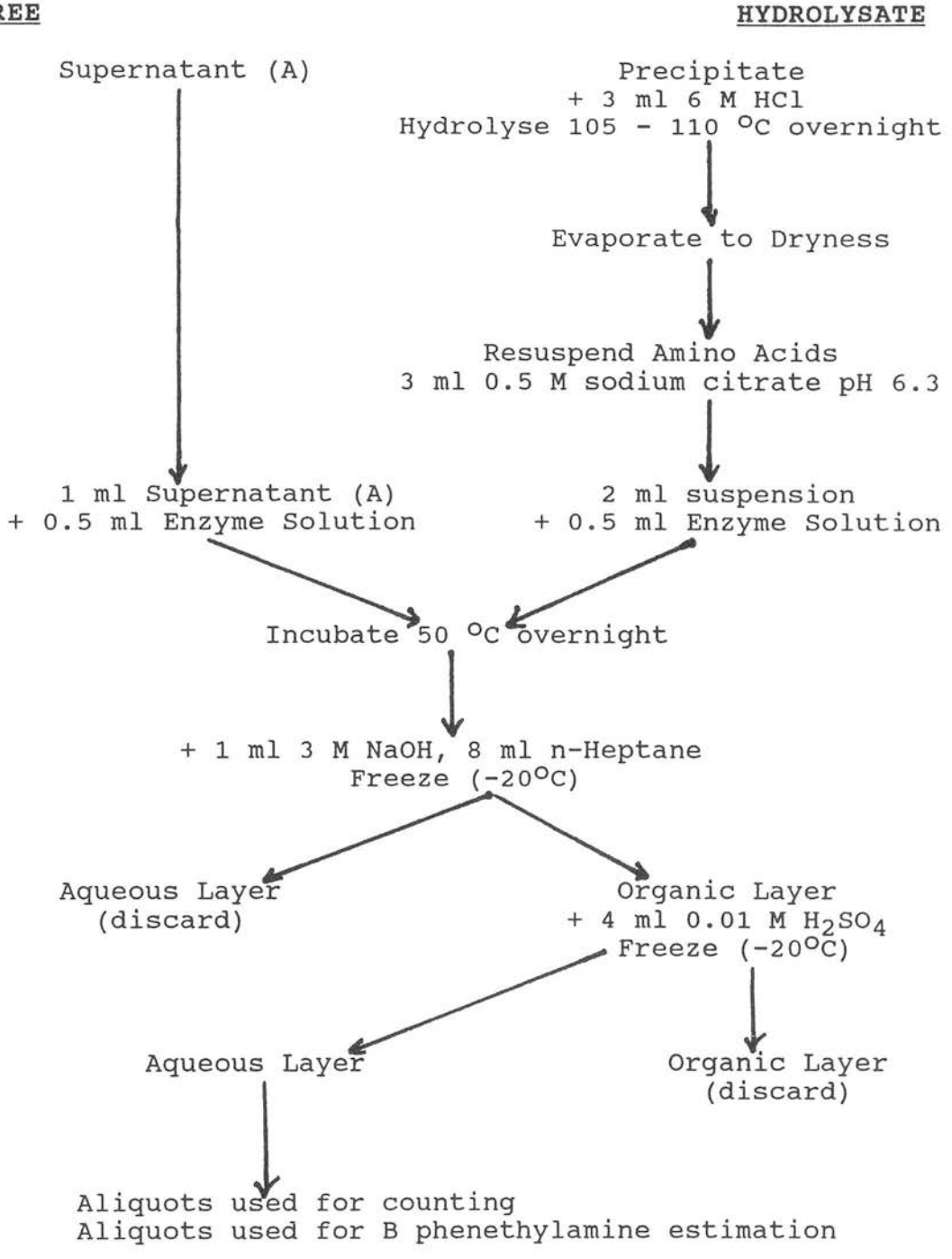

Fig.i. Assay procedure of Protein Synthesis Estimation

Bound Count Rate = 2631.16 dpm*

Bound B-Phenethylamine content = 0.7528 μmoles

Free Count Rate = 4104.62 dpm*

Free B-Phenethylamine content = 0.0082 μmoles

Bound Specific Activity, S_B = 2631.16 x (1/60)/0.7528

= 58.2527 Bq/μmole

Free Specific Activity, S_A = 4104.62 x (1/60)/0.0082

= 8342.7236 Bq/μmole

t = 704 s = 8.148 x 10^{-3} days

Fractional Synthesis Rate, Ks = (S_B x 100)/(S_A x t)

Ks = (58.2527 x 100)/(8342.7236 x 8.148 x 10^{-3})

Ks = 85.6953 %/d

* Corrected for background count rate.

Fig. ii. Example of Fractional Synthesis Rate Calculation.

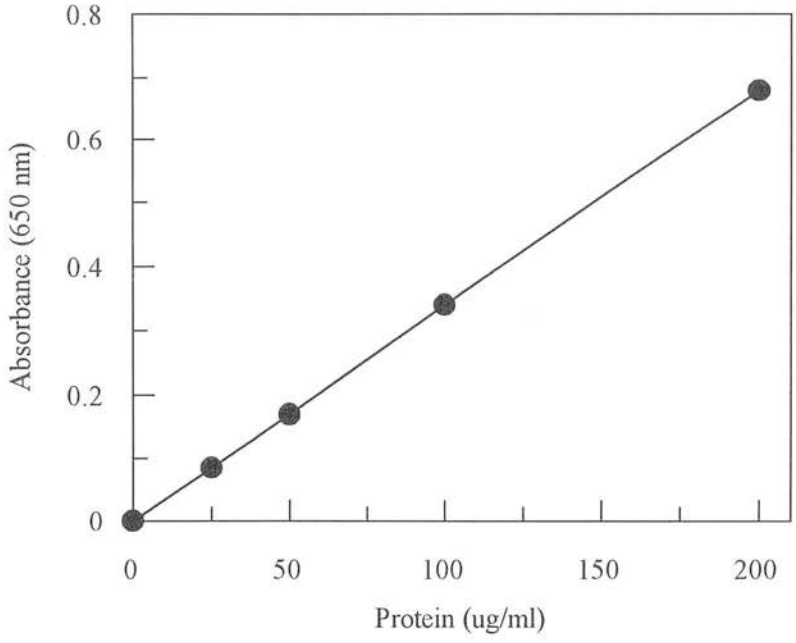

Fig. iii. Standard curve of bovine serum albumin in 0.3 M NaOH.

1. Measure span of O_2 electrode:- Buffer oxygen concentration (before and after Dithionite)

2. Slope of Line on Chart:- pre and post ouabain treatment

Chart set a certain speed cm/hour (A)

10 min recording period = A x (10/60) cm; horizontal difference

$$\% \text{ slope (pre-ouabain)} = \frac{\text{vertical difference}}{\text{horizontal difference}} \times 100$$

This calculation was also carried out on the chart following ouabain treatment.

$$\% \text{ Inhibition of slope} = 1 - \frac{\text{post-ouabain \% slope}}{\text{pre-ouabain \% slope}} \times 100$$

$$\text{nmoles } O_2\text{/min/g Tissue} = \frac{\text{vertical diff. (from 2)}}{\text{span (from 1)}} \times \frac{540^a}{10^b} \times \frac{1}{\text{Weight}^c}$$

This calculation is carried out on the chart for both the pre and post-ouabain periods
Na^+,K^+-ATPase dependent respiration= pre-ouabain - post-ouabain

$$\% \text{ Inhibition} = 1 - \frac{\text{post-ouabain}}{\text{pre-ouabain}} \times 100$$

[a] Oxygen concentration in KRB (180 nmoles/ml)

[b] 10 minutes

[C] Sample Weight

Mucosal respiration recorded as nmoles O_2/min/mg Protein

Fig. iv. Calculation of tissue respiration and the proportion associated with Na^+,K^+-ATPase activity using the rank oxygen electrode.

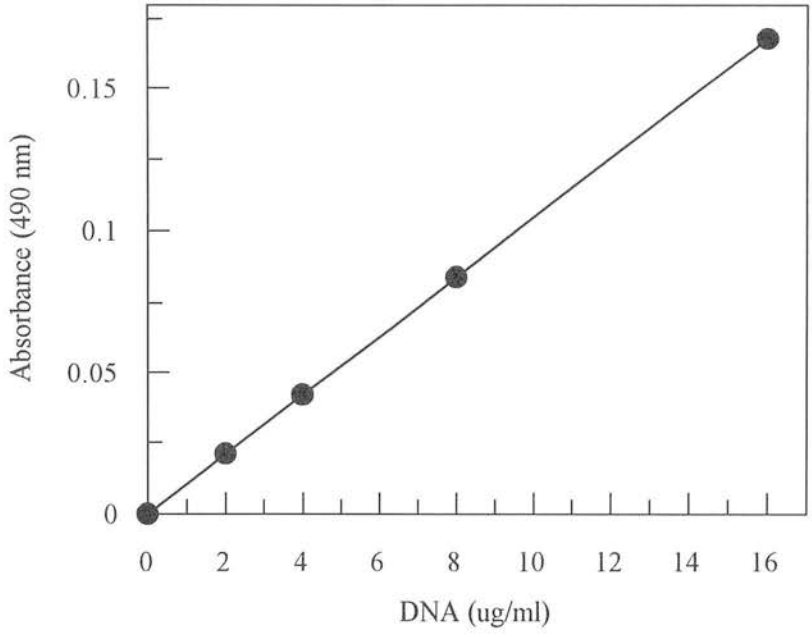

Fig. v. Standard curve of calf thymus DNA.

DNA content from standard curve = 8.0999 μg/ml

DNA content of 2 ml aqueous layer = 16.1998μg/ml

50 ml solution diluted 1:5 to give 2 ml aqueous layer

DNA content of 50 ml solution = 40.4975 μg/ml

= 2.0249 mg

This was provided by 3.5 ml of original 4 ml 0.3 M KOH solution

DNA content of 4 ml solution= 2.3142 mg

DNA Purity = 74.92%*

DNA content = 2.3142 x 0.7492

= 1.7338 mg

Double Stranded DNA = 1.7338/2

= 0.8669 mg

Sample Weight = 0.1836 g

Mammary DNA Content = 0.8669/0.1836

Mammary DNA content = 4.7217 mg/g

* DNA purity checked at 260 nm; extinction of 1.000 = 37 μg/ml

Fig. vi. Calculation of mammary DNA content

APPENDIX 2

Assumptions and Calculations Used in the Development of Nutrient Balances for Dams Offered the 215, 150 and 90 g CP/kg DM Feed.

a: The digestibility of feed and milk protein; 0.95 (Radcliffe *et al.* 1978)

b: The digestibility of feed fat; 0.97 (Chudy *et al.* 1969).

c: The digestibility of feed carbohydrate; 0.95 (Radcliffe *et al.* 1978)

d: Maintenance protein requirement g/d; $10*(0.07^{-0.27})$*(Body Protein) (Emmans *et al.* 1988, Friggens 1990))

e: Efficiency of protein use for maintenance and growth; 0.85 (McDonald *et al.* 1981)

f: Maintenance energy requirement kJ/d; $1630*(0.07^{-0.27})$*(Body Protein) (Emmans *et al.* 1988).

g: Efficiency of carbohydrate use for maintenance; 0.95 (McDonald *et al.* 1981)

h: Efficiency of fat use for maintenance; 0.97 (McDonald *et al.* 1981)

i: Efficiency of protein use for milk protein synthesis; 0.82 (Baldwin *et al.* 1968, 1980) .

j: Efficiency of fat use in milk fat synthesis; 0.89 (39.6 - 4.4/39.6) (Baldwin *et al.* 1980)

k: Efficiency of carbohydrate use for lactose synthesis; 0.95 (Baldwin *et al.* 1980)

l: Heat production from milk protein synthesis; 16.7 kJ/g (Friggens 1990)

m: Heat production from milk fat synthesis; 4.4 kJ/g (Friggens 1990)

n: Heat production from lactose synthesis; 0.9 kJ/g (Friggens 1990)

p: Heat capacity; (1480.1 - 28*(temp ^{o}C))kJ/kg$^{0.75}$/d (Friggens 1990)

q: Supply of endogenous lipid

r: Efficiency of carbohydrate conversion to fat; 0.3 (Friggens 1990)

s: Heat production from carbohydrate conversion to fat; 3.12 kJ/g CHO (Friggens 1990)

t: Supply of endogenous protein.

u, v, w: Energy content of protein, fat and carbohydrate (Appendix 1, Table iii.)

Body Protein: The quantity used for the estimation of maintenance requirements were; For dams offered the 215 gCP/kg DM feed that of group HH (experiment E1), while for the other females values were derived from experiment E3.

Milk Composition: For females offered the 215 and 90 gCP/kg feed, milk composition were those reported in experiment E4, while the milk composition of moderately restricted dams was assumed to be equivalent to that of the high protein group.

Maintenance Energy Requirements: These are assumed to be met primarily by oxidising carbohydrate and if energy requirements exceed that available from carbohydrate then fat is oxidised.

Endogenous Nutrients: Assumed to be used for milk production with the same efficiency as dietary nutrients.

Surplus Nutrients: Assumed to be completely oxidised.

APPENDIX 3

Presented here are the results of a study that was conducted in order to investigate the influence of genotype, diet and stage of lactation on erythrocyte ATPase activity in dairy cattle. This work was carried out prior to the four rat experiments already described in the main part of this thesis.

The influence of genotype, diet and stage of lactation on erythrocyte ATPase activity in dairy cattle. By A. P. PINE and N. S. JESSOP, *Institute of Ecological and Resource Management, University of Edinburgh, Edinburgh EH8 9YL* and J. D. OLDHAM, *Scottish Agricultural College, Edinburgh EH9 3JG*

Evidence suggests that dairy cattle of different milk yield potential may vary in the efficiency with which metabolizable energy is used for maintenance (Taylor *et al.* 1986). As ion transport is a major contributor to metabolic maintenance (Milligan & Summers, 1986), differences in maintenance efficiency may be manifested in different rates of ion transport activity. Using the activity of erythrocyte Na^+,K^+-ATPase (*EC* 3.6.1.3) as a measure of membrane transport we have made an exploratory study on the influence of genotype, diet and stage of lactation on this process. The activity of Mg^{2+}-ATPase (*EC* 3.6.1.4) was also measured.

First lactation heifers from the selected (*n* 18) and control (*n* 14) genetic lines within the Langhill dairy herd were used. Approximately half (*n* 17) received a high forage diet (0·8 kg forage dry matter (DM)/kg total DM) and the remainder a high concentrate diet (*n* 15, 0·55 kg forage DM/kg total DM). Jugular venous blood samples were taken at weeks 8, 19 and 36 post-partum. Erythrocyte membranes were prepared using a modification of the procedure developed by Wheeler & Whittam (1964) and the activities of Na^+,K^+-ATPase and Mg^{2+}-ATPase were measured (Gilbert & Wyllie, 1976).

Erythrocyte ATPase activities (μmol Pi/mg protein per h)

Dietary forage: concentrate ratio . . .	80:20				55:45				Pooled			
	Na^+,K^+-ATPase		Mg^+-ATPase		Na^+,K^+-ATPase		Mg^+-ATPase		Na^+,K^+-ATPase		Mg^+-ATPase	
Week	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
8	0·118	0·028[a]	0·689	0·108[a]	0·163	0·029[a]	0·702	0·139[a]	0·142	0·020[a]	0·697	0·089[a]
19	0·076	0·014[a]	0·334	0·045[b]	0·114	0·019[a]	0·374	0·058[b]	0·096	0·012[b]	0·356	0·038[b]
36	0·045	0·007[b]	0·293	0·025[b]	0·065	0·015[b]	0·241	0·037[b]	0·056	0·009[c]	0·264	0·023[c]

[a,b,c] Values within columns with different superscript letters were significantly different: $P<0·05$.

Both Na^+,K^+- and Mg^{2+}-ATPase activities decreased significantly with stage of lactation ($P<0·05$) suggesting that they reflect increased energy expenditure during early lactation. Diet had no effect on ATPase activities. There was no correlation between enzyme activity and predicted cow genetic index or elements of lactational performance including milk yield and DM intake.

The lack of association in heifers between enzyme activity and genotype suggests that Na^+,K^+-ATPase is not an indicator of lactational potential, although this may change in subsequent lactations.

A.P.P. gratefully acknowledges AFRC support.

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Milligan, L. P. & Summers, M. (1986). *Proceedings of the Nutrition Society* **45**, 185–193.
Taylor, C. S., Thiessen, R. B. & Murray, J. (1986). *Animal Production* **43**, 37–62.
Wheeler, K. P. & Whittam, R. (1964). *Biochemical Journal* **93**, 349–363.